The Function of Mismatch Repair Proteins in Response to DNA Damage caused by Chemotherapeutic Agents

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Artikel1: 5-fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems

Artikel2: O6-methylguanine in the template strand: a futile challenge for human mismatch repair ?

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Zusammenfassung

Um die Effizienz der Fehlpaarungs-Reparatur (mismatch repair, MMR) zu studieren und gleichzeitig die reparierten DNA-Abschnitte sichtbar zu machen, nutzten wir ein spezielles *in vitro*-System: verschiedene Heteroduplex-Substrate mit je einem einzigen fehlgepaarten Basenpaar in der Erkennungssequenz eines Restriktionsenzymes wurden mit Kernextrakt von MMR-profizienten oder –defizienten Zell-Linien und in der Anwesenheit von radioaktiv markierten Nukleotiden inkubiert. Das resultierende Restriktionsmuster gibt Aufschluss über das Reparatur-Ausmass, während die eingebaute Radioaktivität die reparierten Abschnitte in den verschiedenen Fragmenten zeigt.

Ich befasste mich während meiner Doktorarbeit mit der MMR-abhängigen Prozessierung von zwei bestimmten DNA-Läsionen, welche beide durch chemotherapeutische Substanzen verursacht werden: O^6-Methylguanin (^{m}G) und 5-Fluorouracil (5-FU).

^{m}G entsteht als geringfügige Untermenge bei der Behandlung von Zellen mit S_N1-methylierenden Mitteln, wie zum Beispiel *N*-methyl-*N'*-nitro-*N*-nitrosoguanidin (MNNG) oder das in Kliniken verwendete Temozolomid (TMZ). ^{m}G ist nicht nur mutagen, sondern auch zytotoxisch und es konnte gezeigt werden, dass ein funktionales MMR-System für die ^{m}G-induzierte Zytotoxizität verantwortlich ist: im Gegensatz zu MMR-profizienten Zellen tolerieren MMR-defiziente Zellen nämlich den Methylierungs-Schaden. Der genaue Mechanismus des durch ^{m}G verursachten Zelltods ist nicht bekannt, doch es existieren dazu mehrere Modelle. Die sogenannte “futile repair”-Hypothese (“vergebliche Reparatur”) geht davon aus, dass die MMR-Maschinerie ^{m}G erkennt und versucht, den Schaden zu beheben. Da die Läsion aber im Matrizenstrang liegt und das MMR-System nur den neu-synthetisierten Strang reparieren kann, laufen vergebliche Exzision/Resynthese-Zyklen gegenüber des Schadens ab. Als Konsequenz dieser nicht erfolgreichen Reparaturversuche entstehen möglicherweise Doppelstrangbrüche, welche schlussendlich zum Zelltod führen könnten. Eine Alternative zu dieser Hypothese ist ein Modell, welches besagt, dass MMR-

Faktoren die nicht reparierbare Modifikation erkennen und direkt Apoptose einleiten.

5-FU dagegen ist die klassische chemotherapeutische Substanz zur Behandlung von kolorektalem Krebs (CRC). Dieser Anti-Metabolit kann sowohl in RNA als auch in DNA eingebaut werden und hemmt zudem die Thymidylat-Synthase (TS), was eine Verlangsamung der DNA-Replikation zur Folge hat. Das MMR-System ist auch in die 5-FU-Prozessierung involviert: verschiedene Studien haben bewiesen, dass Patienten mit MMR-defizientem CRC keinerlei Wirkung auf Chemotherapien mit 5-FU zeigen und die Beobachtungen konnten *in vitro* bestätigt werden. Diese Resultate lassen den Schluss zu, dass das MMR-System eine wichtige Rolle in der 5-FU-basierten Krebstherapie spielt. Auf der anderen Seite ist 5-FU aber auch ein Substrat für alle bekannten menschlichen Uracil-DNA-Glykosylasen: uracil-DNA glycosylase (UNG), thymine-DNA glycosylase (TDG), single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1) und methyl-CpG binding domain protein 4 (MBD4).

Das erste Projekt meiner Doktorarbeit widmete sich der Frage, ob diese vergeblichen Reparatur-Zyklen gegenüber ^{m}G tatsächlich stattfinden. Gleichzeitig richteten wir unser Augenmerk auf die Entstehung von abweichenden DNA-Zwischenprodukten, wie zum Beispiel einzelsträngige Regionen, und untersuchten das Verhältnis der gegenüber ^{m}G eingebauten T- und C-Basen. Unsere Resultate zeigen, dass das MMR-System ^{m}G-enthaltende DNA-Substrate *in vitro* erkennt und prozessiert. In Übereinstimmung mit früheren Ergebnissen konnten wir bestätigen, dass das hMLH1/hPMS2-Heterodimer für die 5'-gerichtete Fehlpaarungs-Reparatur in unserem *in vitro*-System entbehrlich ist. Die Affinität von hMutSα für ein G/T-Fehlpaar in Bandshift-Experimenten ist erheblich grösser als für ^{m}G/T oder ^{m}G/C, was die tiefere Reparatur-Effizienz in unserem MMR-System *in vitro* erklärt. ^{m}G/T- und ^{m}G/C-Fehlpaare, bei welchen sich ^{m}G im eingeschnittenen Strang befindet, stellen schwache Substrate dar und werden mit der gleichen Ineffizienz prozessiert und repariert. Pulse-Chase-Experimente zeigten keine wesentliche Exzision von radioaktiv markiertem dAMP und so konnte weder in 5'- noch in 3'-Substraten eine MMR-abhängige Abnahme der Radioaktivität aufgrund vergeblicher Reparatur-Zyklen beobachtet werden. Als Alternative

versuchten wir das bei diesen mutmasslichen Reparatur-Zyklen exzisierte radioaktiv markierte dAMP mittels Dünnschicht-Chromatographie aufzutrennen. Da der von uns verwendete Kernextrakt aber die intrinsische Fähigkeit besass, dATP zu hydrolysieren, wurden die Resultate von dieser Hintergrund-Aktivität überdeckt. Aufgrund der schwachen Erkennung von ^{m}G-enthaltenden Substraten durch unser *in vitro*-MMR-System konnten weder Einzelstrang-Lücken detektiert noch klare Aussagen über den Einbau von C oder T gegenüber ^{m}G gemacht werden. Nichtsdestotrotz bestätigte eine Forschungs-Gruppe im Sommer 2006 die Existenz der vergeblichen Reparatur-Zyklen gegenüber ^{m}G *in vitro* und lieferte so einen ersten biochemischen Beweis für die “futile repair”-Hypothese.

Das zweite Projekt konzentrierte sich auf verschiedene Fragen im Zusammenhang mit der 5-FU-Prozessierung *in vitro*. Als erstes überprüften wir die Fähigkeit unseres *in vitro*-MMR-Systems, 5-FU-Substrate zu reparieren. In einem zweiten Schritt immuno-dezimierten wir eine Glykosylase nach der anderen aus dem Kernextrakt, um schliesslich die Verantwortliche(n) der 5-FU-Reparatur zu finden. In einem letzten Schritt untersuchten wir die Kompetition zwischen MMR und Glykosylasen auf dem 5-FU-Substrat. Wir konnten zeigen, dass sowohl G/5-FU- als auch A/5-FU-Fehlpaare in zirkulären DNA-Substraten von der MMR in menschlichen Zellextrakten angegangen werden. Im Übrigen wird G/5-FU durch die Basen-Exzisions-Reparatur sehr effizient zu G/C repariert. In diesem Prozess spielt TDG eine übergeordnete Rolle. Kompetition zwischen den beiden Reparatursystemen konnte beobachtet werden.

Methylierende Substanzen und 5-FU sind feste Bestandteile etablierter Chemotherapien im Kampf gegen eine Vielzahl von Krebsarten. Es ist deshalb von grosser Wichtigkeit, den genauen molekularen Mechanismus des jeweiligen Zellgiftes zu kennen, um endogene und erworbene Resistenzen gegenüber chemotherapeutischen Mitteln möglichst zu vermeiden. Unsere *in vitro*-Resultate rücken die Glykosylasen als mögliche Zielproteine in der 5-FU-basierten Krebstherapie in ein neues Licht und weisen vielleicht den Weg für eine erfolgreichere Behandlungsstrategie.

Summary

Mismatch repair (MMR) efficiency and repair tract visualization can be studied in an *in vitro* MMR assay as follows: different heteroduplex substrates containing a single mismatch within a restriction endonuclease site are incubated with nuclear extract from either MMR-proficient or –deficient cell lines, in the presence of radiolabeled nucleotides. The resulting restriction pattern gives information about the repair extent and the incorporated radioactivity in the different fragments reveals the repair tracts.

I've studied the MMR-dependent processing of two particular DNA lesions induced by chemotherapeutic substances: O^6-methylguanine (^{m}G) and 5-fluorouracil (5-FU).

^{m}G arises as a minor subset in cells treated with S_N1-type methylating agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or its clinical counterpart temozolomide (TMZ). This lesion, besides being mutagenic, causes cell death and since MMR-deficient cells exhibit a methylation-tolerant phenotype, MMR has been shown to be instrumental in mediating the observed ^{m}G-induced cytotoxicity. But the exact mechanism by which ^{m}G kills the cells remains elusive, although a number of models have been proposed. One model, the futile repair hypothesis, suggests that the MMR system will attempt to process the ^{m}G lesions, but as the modified base is in the template strand, and as MMR is exclusively targeted to the newly synthesized strand, several rounds of futile excision/resynthesis cycles are the consequence. These unsuccessful repair events may end up in double-strand breaks and thus lead to cell death. An alternative to this model suggests that MMR factors recognizing the unrepairable lesion directly signal apoptosis.

5-FU, on the other hand, is the agent of choice in the treatment of colorectal cancer (CRC) and this antimetabolite can be incorporated into both RNA and DNA. Furthermore, it inhibits thymidylate synthase (TS), thereby slowing down DNA replication. MMR is involved in 5-FU-processing as well: patients with MMR-deficient CRCs were reported not to benefit from 5-FU-based chemotherapy, and these findings appear to be confirmed by *in vitro* studies. This implies a role for the MMR system in the response to 5-FU treatment. But 5-FU is also a substrate for all four known human uracil DNA glycosylases:

uracil-DNA glycosylase (UNG), thymine-DNA glycosylase (TDG), single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1) and methyl-CpG binding domain protein 4 (MBD4).

The first project of my PhD study mainly addressed the question whether or not futile repair cycles are taking place opposite ^{m}G. Concomitantly, we looked in more detail at the formation of possible aberrant DNA intermediates such as single-stranded regions and examined the T:C ratio of incorporation opposite ^{m}G. Our results show that the mismatch repair machinery recognizes and processes DNA substrates containing ^{m}G *in vitro*. In agreement with earlier findings, hMLH1/hPMS2 heterodimer is not essential for 5'-directed MMR in our *in vitro* assays. The affinity of hMutSα for the G/T mispair in bandshift experiments is substantially greater than for ^{m}G/T or ^{m}G/C, consistent with lower repair efficiency in our *in vitro* mismatch repair assays. ^{m}G/T and ^{m}G/C mismatches (lesion in the nicked strand) are processed and repaired with equally low efficiency. Pulse chase experiments did not show a significant excision of the radiolabeled dAMP, and therefore no visible MMR-dependent decrease of radioactivity due to futile repair cycles were detected, neither in the 5'- nor in the 3'-substrates. Thin layer chromatography assays to separate the radiolabeled dAMP, which was expected to be excised during the putative iterative repair cycles, could not overcome the problem due to the hydrolytic activity of the used nuclear extracts masking the result. Because of the weak recognition of our ^{m}G-containing substrates by MMR, neither gaps nor non-ambiguous C- or T-incorporation opposite the lesion could be clearly confirmed with our system. However, in summer 2006, another group reported the existence of iterative excision opposite ^{m}G *in vitro*, thus confirming the futile repair model.

In the second project, we focused on different questions concerning the 5-FU processing in the above-described *in vitro* assay. First, we checked repair capability of the MMR machinery on 5-FU substrates. Second, we immunodepleted one glycosylase after the other from the nuclear extracts to find the one(s) responsible for 5-FU repair. In the last step, we looked at the competition between MMR and glycosylases on the 5-FU substrate. We could show that both G/5-FU and A/5-FU base pairs incorporated into circular DNA substrates are addressed by the MMR system in extracts of human cells. In

addition, the former substrate is very efficiently repaired to G/C by base excision repair (BER). In this processing, TDG plays the predominant role. Competition between the two repair pathways could be observed.

Since methylating agents and 5-FU are inherent parts in approved chemotherapies against a variety of cancers, it is of maximum importance to understand the exact molecular mechanism of the appropriate drug to avoid endogenous and acquired resistance. Our *in vitro* results may point to the glycosylases as an additional class of target proteins, which deserve closer consideration in order to improve 5-FU-based regimens.

1. Introduction

DNA is constantly subjected to alterations by exogenous and endogenous sources. Ionizing and UV radiation, or carcinogens contained in food, cigarette smoke and particular environments rank among the former, cellular metabolites causing hydrolysis or methylation and active oxygen species constitute the latter [1]. To deal with this permanent threat and to avoid consequent genomic instability, nature armed living cells with a series of sophisticated defense mechanisms: damage repair, damage tolerance and the possibility of checkpoint activation (**Fig. 1**).

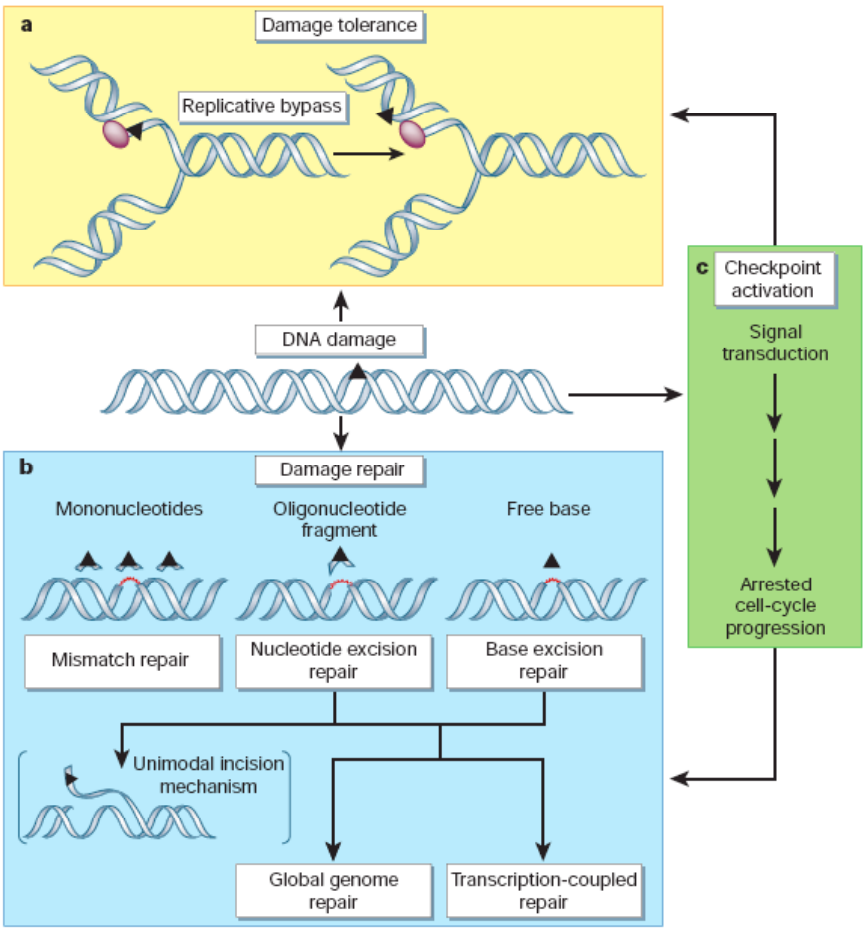

Figure 1 Responses to DNA damage. DNA damage (illustrated as a black triangle) results in either repair or tolerance. **a** During damage tolerance, damaged sites are recognized by the replication machinery before they can be repaired, resulting in an arrest that can be relieved by replicative bypass (translesion DNA synthesis). **b** DNA repair involves the excision of bases and DNA synthesis (red wavy lines), which requires double-stranded DNA. Mispaired bases, usually generated by mistakes during DNA replication, are excised as single nucleotides during mismatch repair. A damaged base is excised as a single free base (base excision repair) or as an oligonucleotide fragment (nucleotide excision repair). Such fragments are generated by incisions flanking either side of the damaged base. Nucleotide excision repair can also transpire in some organisms by a distinct biochemical mechanism involving only a single incision next to a site of damage (unimodal incision). **c** The cell has a network of complex signalling pathways that arrest the cell cycle and may ultimately lead to programmed cell death (from [2]).

DNA repair encompasses a multitude of metabolic processes, which come into play in dependence of the respective kind of damage.

Four broad repair categories can be distinguished, including direct reversal of damage, excision of the damaged region, followed by precise replacement (base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), repair of interstrand cross-links), double-strand break repair and damage tolerance (damage bypass).

The following section will be confined to the particular subset of DNA repair mechanisms used in my studies, namely direct repair, BER and MMR.

2. DNA repair mechanisms

2.1 Direct repair

In the simplest type of DNA repair, the damaged base is repaired directly, e.g. dealkylated, by a one-step mechanism, rather than being excised and replaced by the correct one.

2.1.1 Photolyase

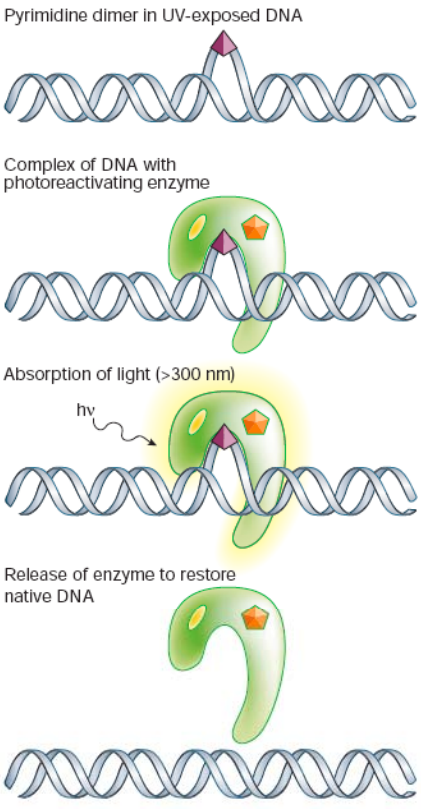

Figure 2 Photoreactivation reverses DNA damage. DNA exposed to ultraviolet (UV) radiation results in covalent dimerization of adjacent pyrimidines, typically thymine residues (thymine dimers), illustrated here as a purple triangle. These lesions are recognized by a photoreactivating enzyme, which absorbs light at wavelengths >300nm (such as fluorescent light or sunlight) and facilitates a series of photochemical reactions that monomerize the dimerized pyrimidines, restoring them to their native conformation (taken from [2]).

UV radiation promotes the formation of a cyclobutyl ring between adjacent thymine residues on the same DNA strand to form an intrastrand thymine dimer. Similar cytosine and thymine-cytosine dimers are likewise formed, but at lesser rates. Such pyrimidine dimers locally distort DNA's base paired structure so that it can form neither a proper transcription nor replication template. By using blue-light photons as an energy source, photolyases, monomeric proteins of 55-65 kDa with two chromophore cofactors, split these

aberrant structures. There are two types of photolyases, one that repairs cyclobutane pyrimidine dimers (photolyase) and the other that repairs (6-4) photoproducts (6-4 photolyase) [3-6]. In 1949, this so-called photoreactivation was the first DNA repair process to be discovered (**Fig. 2**).

2.1.2 O^6-methylguanine-DNA methyltransferase (MGMT)

Many carcinogens in the environment [7], tobacco smoke [8] and food [9], in addition to endogenous metabolic products [10], methylate DNA. One of the consequences is the formation of the highly mutagenic and cytotoxic lesion O^6-methylguanine. It is repaired by MGMT (also referred to as O^6-alkylguanine-DNA alkyltransferase, AGT), which removes the methyl group from guanine in a single step, transferring it to an internal cysteine residue. This methyl-group transfer inactivates MGMT and restores guanine in the DNA ([11], **Fig. 3**). In contrast to the above-described photolyases, which are not found in *E. coli*, yeast and mammals, O^6-methylguanine-DNA methyltransferases are nearly universally distributed in nature. For example, *E. coli* expresses two genes encoding proteins of this type, one is inducible (*ada*) and one is constitutively expressed (*ogt*). The Ada protein not only repairs methylated bases but also regulates the so-called adaptive response, involving the induction of the *ada*, *alkA, alkB* and *aidB* genes upon exposure to methylating agents [12].

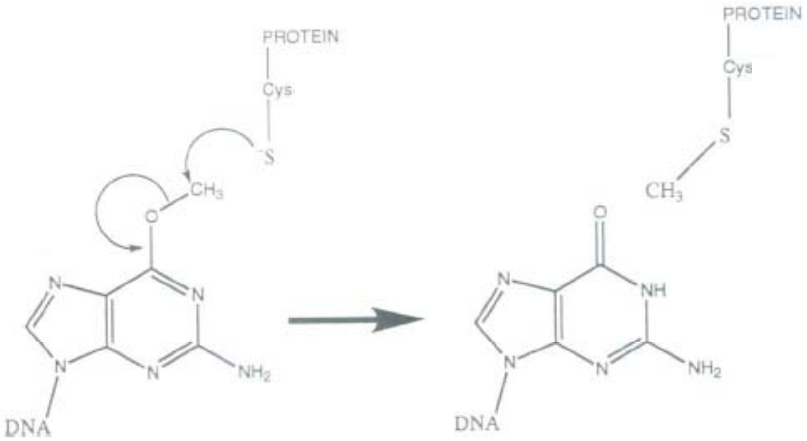

Figure 3 A line drawing of the overall methyl-transferase suicide repair reaction. This shows the methyl group being transferred from the O^6-position on a guanine in DNA to the sulfur side-chain of the active site thiol (from [13]).

2.1.3 AlkB

AlkB, like Ada, is also a damage-reversal protein and was recently shown to be an α-ketoglutarate-Fe(II)-dependent dioxygenase, which repairs 1-methyladenine and 3-methylcytosine in DNA by oxidative demethylation ([14], **Fig. 4**).

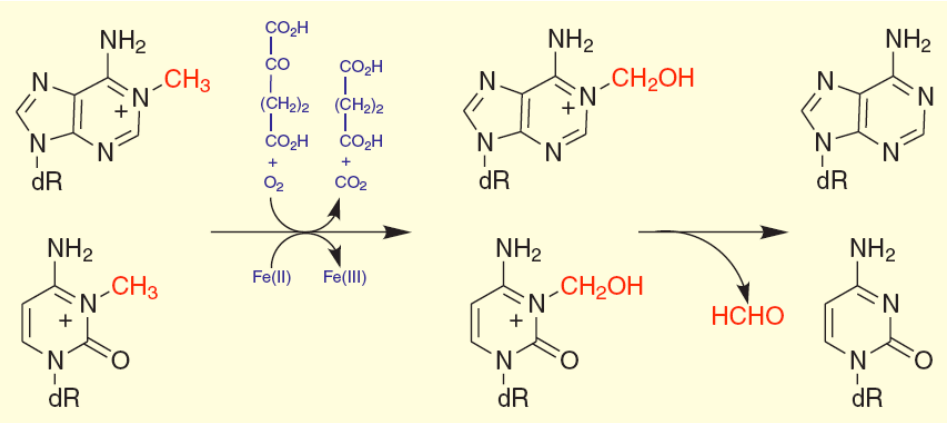

Figure 4 AlkB-mediated demethylation of *N1*-methyladenine and *N3*-methylcytosine. The methyl groups are converted to hydroxymethyl moieties, which are spontaneously lost in the form of formaldehyde to regenerate the unmodified bases (the aberrant methyl groups and their oxidation products are shown in red). Note that the cofactors (blue) are consumed during the reaction in stoichiometric amounts (from [15]).

2.2 Base excision repair (BER)

2.2.1 Overview

As the name implies, the initial step in BER is the removal of an aberrant or damaged base, and this job is carried out by specialized enzymes, DNA glycosylases. While several of them have very narrow substrate specificity, most glycosylases remove more than one structurally different damaged or mispaired base, thereby addressing modifications like oxidation, deamination or alkylation. It has been possible to reconstitute the BER process using purified proteins from bacteria to mammals, and it is fundamentally conserved throughout evolution [16-21]. After recognition of the suspect base, glycosylases remove it by cleaving the N-glycosidic bond between the base and the sugar-phosphate backbone of the DNA. The resulting abasic site is subsequently processed by one of two BER pathways: in short-patch BER, removal of the aberrant base is followed by strand cleavage 5' to

the formed abasic site by an AP endonuclease (APE1 in humans). In the next step, the baseless sugar-phosphate is replaced by polymerase β, attaching a single nucleotide to the newly generated 3'-OH end and removing the sugar-phosphate moiety by its lyase activity [21]. DNA ligase III, which interacts with polymerase β through the XRCC1 protein (X-ray cross-complementation protein 1), seals the nick to restore the original DNA sequence [17]. In an alternative mechanism, long-patch BER, APE1 makes the 5' incision to the AP site, and then the combination of DNA polymerase δ/ε, RFC, PCNA, and FEN1 displaces the strand 3' to the nick to produce a flap of 2-10 nucleotides, which is excised at the junction of the single- to double-strand transition by the flap-endonuclease FEN1. Concomitantly, repair synthesis of the eliminated patch is carried out by polymerase δ/ε, RFC, PCNA or polymerase β [20-22]. Finally, the nick is ligated by DNA ligase I. The short-patch repair pathway presumably predominates and, in certain cases, appears to be used exclusively, for example when base excision is mediated by DNA glycosylases/AP lyases (**Fig. 5**) [23]. Long-patch BER may serve as a back-up pathway and could be important for the removal of modified abasic sites that are resistant to the AP lyase activity of polymerase β [18].

2.2.2 Glycosylases

Mechanistically, glycosylases can be divided into two classes: (1) monofunctional DNA glycosylases, and (2) bifunctional DNA glycosylases/AP lyases. The former cleave the glycosidic bond using water as a nucleophile and generate apurinic or apyrimidinic (AP) sites (**Fig. 6**). The latter utilize an amino group of the enzyme as a nucleophile to form a Schiff's base intermediate, which undergoes enzyme-catalyzed β-elimination cleaving the phosphodiester bond 3' from the abasic site [24].

The four known monofunctional uracil-DNA glycosylases in humans, namely UNG (uracil-DNA glycosylase), TDG (thymine-DNA glycosylase), MBD4 (methyl-CpG binding domain protein 4) and SMUG1 (single-strand selective monofunctional uracil-DNA glycosylase 1) accompanied me during the studies of my thesis and therefore I will turn my attention in particular to this quadripartite team of UDGs.

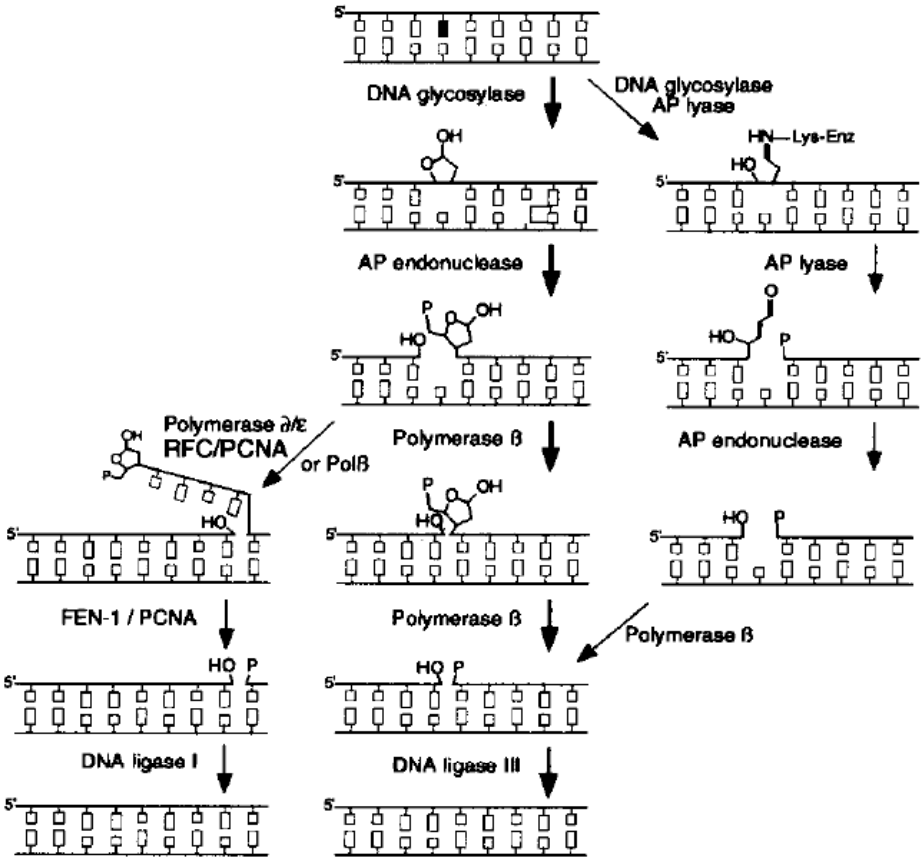

Figure 5 Overview of base excision repair pathways: The short-patch BER pathway shown in the center is predominant. Excision of a damaged base by a DNA glycosylase or spontaneous base loss generates an abasic site, which is processed to a single-strand break by an AP endonuclease. The repair synthesis of one nucleotide and the excision of the abasic site is brought about by polymerase β, and DNA ligase III seals the remaining nick to restore the original DNA sequence. The short-patch pathway involving a bifunctional DNA glycoylase/AP lyase is outlined on the right. This pathway also requires APE1, Pol β and DNA ligase III, with the difference that Pol β is not involved in the removal of the baseless sugar-phosphate. In the long-patch repair pathway, Pol δ/ε supported by the replication factors RFC/PCNA, makes a repair tract of 2±6 bases and FEN-1, supported by PCNA, excises the overhanging oligonucleotide. DNA ligase I then seals the nick (from [25]).

All of them display affinity for bases that arise through deamination of cytosine and/or 5-methylcytosine residues. While three of them (UNG, SMUG1 and TDG) belong to the UDG family of DNA glycosylases, possess the same fold, and have probably evolved from a common ancestor [26], MBD4 is a member of the helix-hairpin-helix (HhH) family of DNA glycosylases [25]. All DNA glycosylases flip the target nucleotide out of the double helix and into an active site pocket of the enzyme where catalysis takes place. This pocket consists of aromatic residues that stack against the base, polar side chains that make specific hydrogen bonds, and

hydrophobic residues that control shape and size of the pocket. The mechanism of glycosidic bond cleavage is very similar for most enzymes and involves the attack of an activated nucleophile at the anomeric position to displace the damaged base [25].

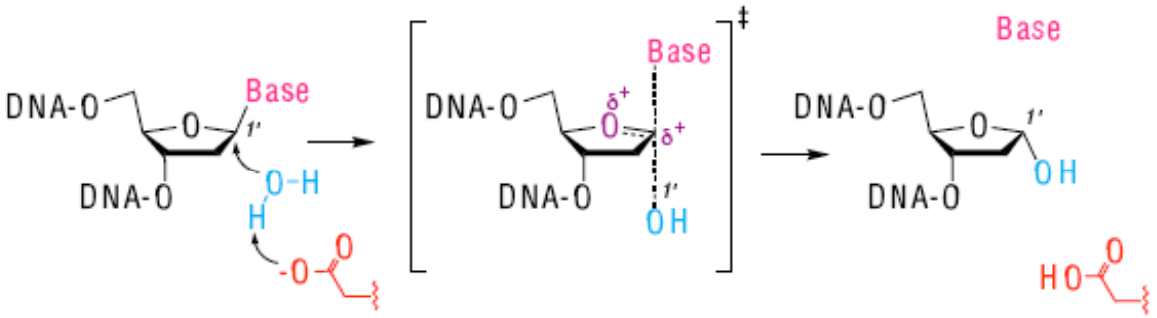

Figure 6 The general mechanism of monofunctional DNA glycosylases. The structure indicated in brackets represents the transition state, in which substantial partial positive charge accumulates on the 2′-deoxyribose ring, especially at the positions denoted by δ+.

2.2.2.1 Uracil-DNA glycosylase (UNG)

Uracil in DNA can be introduced via two mechanisms, deamination of cytosine and misincorporation of dUMP during replication. Whereas U/G mispairs resulting from deamination of cytosines are mutagenic, misincorporation of dUMP during replication leads to U/A mispairs, which, although not miscoding, may produce cytotoxic and mutagenic AP site intermediates due to repair events. To prevent cytotoxic and mutagenic effects of the above lesions, all living organisms express uracil-DNA glycosylases.

Uracil-DNA glycosylase activity was first detected in 1974 by Tomas Lindahl in extracts of *E. coli* [27], and has since been isolated from various other sources including yeast [28], mammals [29-31] and large eukaryotic DNA viruses [32]. Eukaryotic cells have both a nuclear and a mitochondrial form of uracil-DNA glycosylase [33-35] and it is assumed that the mitochondrial splice variants of many eukaryotic DNA glycosylase mRNAs protect the mitochondrial DNA against damage resulting from its proximity to the electron transport system [36]. The evolutionarily highly conserved human uracil-DNA glycosylase was cloned in 1989 and it could be shown that the *in vitro* expressed protein not only exhibited uracil-DNA glycosylase activity, but could also restore wild-type phenotype when expressed in *E. coli ung* mutants [37, 38]. Since then, UNG has been extensively studied, and its structure

and molecular mechanism of catalysis and specificity established [39-42]. The enzyme removes uracil *in vitro* in the order of preference ssU > U/G > U/A [43], and, in addition, excises 5-hydroxyuracil, isodialuric acid and alloxan, although at very low rate compared with uracil [44]. Several lines of evidence ascribe a major role in postreplicative removal of misincorporated uracil in mammalian cells to UNG2 by showing its interaction with RPA and PCNA and colocalization with these two proteins to replication foci [45]. Studies with *Ung*$^{-/-}$ mice underline these results by showing significant accumulation of dUMP in the DNA of the knockout cells [46]. Since neither overt phenotype abnormalities, nor predisposition to cancer could be observed [46], the finding that the increase in uracil content of genomic DNA in the knockout cells had only a marginal effect on the spontaneous mutation frequency suggested that murine UNG is involved primarily in the removal of uracil incorporated during replication. Therefore it was speculated that in higher eukaryotes, a redundant activity such as SMUG1 could instead be the responsible glycosylase for excision of deaminated cytosines [47]. However, bacterial and yeast *ung* mutants display a mutator phenotype as they are unable to repair deaminated cytosines [48, 49] and Kavli *et al.* could show that recombinant nuclear UNG2 likely is the major nuclear enzyme for removal of deaminated cytosines in both double- and single-stranded DNA *in vitro* [50]. The contribution of UNG2 to repair of deaminated cytosines therefore remains controversial and demands further investigation.

2.2.2.2 Thymine-DNA glycosylase (TDG)

Cytosine and 5-methylcytosine are subject to spontaneous deamination. While deamination of cytosine produces uracil, which can be readily removed by uracil-DNA glycosylase [51], deamination of 5-methylcytosine yields thymine, thus generating a G/T mispair. In an attempt to prove that such mismatches are corrected in favor of guanine, Brown and Jiricny introduced specific G/T mispairs into the genome of SV40 and determined the fate of the mismatched bases in African Green Monkey (CV1) cells [52]. Indeed, a G/T directed repair activity that efficiently replaced the T with a C could be observed. The subsequent purification of a G/T binding and processing enzyme from nuclear extract of HeLa cells and the molecular cloning of the respective cDNA led to the discovery of the human thymine-DNA

glycosylase [53-56]. Orthologs in bacteria [57], yeasts, insects [58] and frogs are known, all of them belonging to the MUG branch superfamily of monofunctional uracil-DNA glycosylases that share a common α/β-fold structure [26]. Although the human TDG is best known for its ability to remove T from a T/G mismatch, MUG proteins of different origin were shown to have rather broad substrate spectra with U mispaired to G being the common, most efficiently processed physiological DNA lesion [57-60]. Derivatives of U with modifications or substituents at the 5-carbon position (e.g. 5-bromouracil, 5-fluorouracil) turned out to be very efficiently and universally processed substrates as well [58]. Moreover, the MUG protein family act also on DNA lesions as divergent as ethenoadducts [58, 61, 62], deaminated purines [58] and thymine glycol [63]. Studies of the three-dimensional structure of the *E. coli* Mug protein revealed some unique features: while UNG and SMUG enzymes establish highly specific hydrogen bonds with the uracil residue [64-66], Mug forms a large hydrophobic pocket with space for a variety of substrates as described above, without contacting the base to be hydrolyzed [66], but, instead, interacts with the complementary DNA strand opposite the target base. Thus, Mug, and probably the entire MUG family, uses the complementary base for substrate discrimination, while other UDGs establish specific contacts with the substrate base. Apparently, MUG proteins have evolved with little selective pressure, so that, in accordance with the specific needs of individual species, enzymes or enzyme complexes with rather distinct functionalities could develop.

It is also noteworthy that human TDG was shown to bind to an AP-site opposite G with an affinity that is higher than that to any of its preferred substrates (e.g. G/U or G/T mismatches [67, 68]). Thus, the available experimental evidence suggests that a conformational change, involving the N-terminal domain of TDG, provides unspecific DNA interactions that facilitate processing of a wider spectrum of substrates at the expense of enzymatic turnover. SUMOylation then reverses this structural change in the product bound TDG (**Fig. 7**) [69].

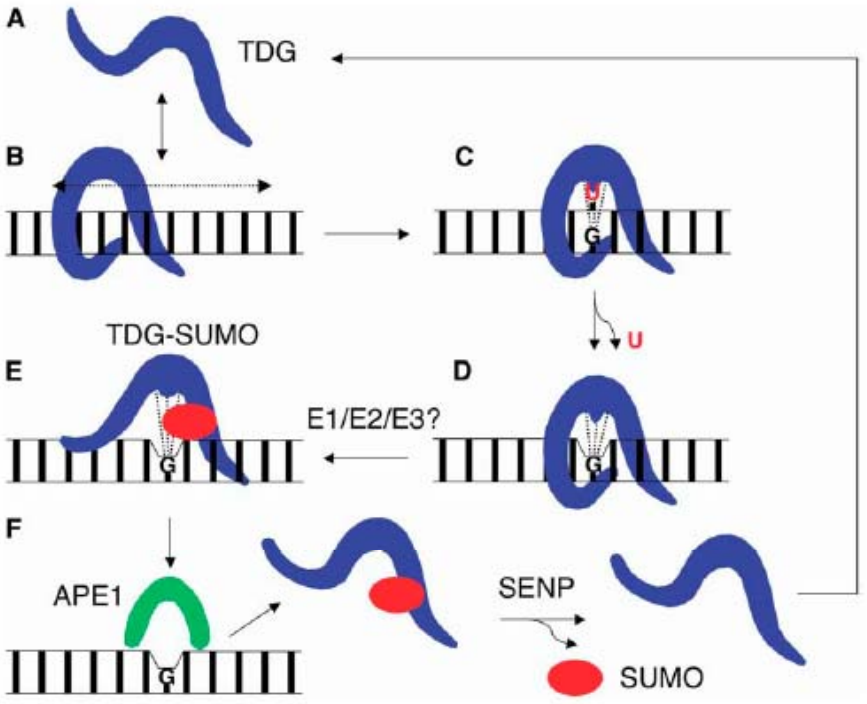

Figure 7 A model for dynamic DNA interactions associated with base excision by TDG. Experimental evidence predicts four different conformational states of TDG.
A DNA-free TDG has an open conformation.
B Upon encountering a DNA molecule, the N-terminus forms a closed structure with the catalytic CORE, mediating nonspecific DNA binding. This mode of interaction may allow TDG to slide along the DNA, to dissociate and reassociate with DNA, in search of a potential substrate.
C and D G•U- or G•APsite bound TDG reflects a third conformational state, where an amino acid wedge penetrates the DNA duplex and forms specific contacts with the guanine opposite. The nonspecific and the specific DNA contacts now cooperate to prevent the dissociation of TDG from the substrate.
E SUMO modification of TDG then induces a fourth conformational state, neutralizing the nonspecific DNA interactions of the N-terminus and facilitating the dissociation of the enzyme from the AP site.
F APE1 gains access to the AP site and continues the BER process. Demodification by SENP proteins allows recycling of TDG and SUMO (from [69]).

Concerning the biological functions of TDG, interesting considerations can be taken into account. As discussed above, UNG-deficient cells accumulate significant amounts of dUMP in their DNA despite the presence of TDG, MBD4 and SMUG1 [46]. These A/U mispairs arising during replication are reported to be poor substrates for TDG [60], arguing strongly against a major contribution of the enzyme to the elimination of U that gets misincorporated opposite A. A direct replication-associated function of TDG is further excluded by the fact that the protein is actively degraded by the proteasome pathway at the G1/S boundary of the cell cycle and then remains undetectable during the entire S-phase (Ulrike Hardeland *et al.*, manuscript submitted). To keep the diverged features of MUG proteins in mind: in fission yeast *S. pombe*, unlike in mammalian cells, the replicative uracil-DNA glycosylase Ung1

acts synergistically with the TDG ortholog Thp1 to eliminate U that gets misincorporated during DNA replication.

As mentioned above, the fact that inactivation of UNG in mouse did not significantly alter the mutation frequency argues for G/U correction being achieved by redundant activities. But since the C → T transition frequency at *hprt* locus of Ung-deficient mouse cells increases synergistically when *Smug1* is silenced by siRNA, both UNG2 and SMUG1 are good candidates [70]. However, TDG is likely to act on deaminated cytosine as well, since it is highly active on a G/U substrate and is expressed in most mammalian cell types.

With four enzymes competing for uracil excision in mammalian cells, the situation is complex and implies functional separation. Hence, whereas UNG and SMUG1 may act more globally on genome repair, the G/U processing by TDG (and MBD4) may be confined to certain areas of the genome and/or to specific physiological states of the cells.

Already in 1992, a physical interaction of mouse TDG with the transcription factor c-Jun has been reported [71]. Since then, a multitude of physical and functional associations between TDG and transcription factors could be observed [72-78] and the question arises whether and how the DNA glycosylase activity of TDG can be reconciled with a role in gene regulation in a plausible functional concept. Possibly, the specific recruitment to gene regulatory elements through the interaction with transcription factors would allow TDG to interrogate the integrity of methylated and unmethylated CpG sequences [79].

In embryonic development, TDG probably has a non-redundant essential function, as homozygous *Tdg* null-embryos lose viability at midgestation (Primo Schär *et al.*; Tetsuya Ono *et al*., manuscripts in preparation).

As an apparent jack-of-all-trades, TDG presumably also plays a critical role in cancer therapy. 5-fluorouracil (5-FU), an antimetabolite used in chemotherapy against a wide range of human cancers, is an excellent substrate for the MUGs [58]. Surprisingly, inactivation of TDG in mouse embryonic fibroblasts causes hyperresistance to moderate doses of 5-FU (Christophe Kunz and Primo Schär, manuscript in preparation). Thus, TDG contributes significantly to the DNA-directed cytotoxicity of 5-FU. We can strongly confirm this finding with our *in vitro* results,

where we observe a predominant role of TDG in the removal of 5-FU from 5-FU/G mismatches on circular heteroduplex substrates (see ***Results***, Fischer *et al*., manuscript submitted). It remains to show the correlation between TDG activity in human tumors and the response to 5-FU-based chemotherapy.

Finally, the G/T processing function of TDG may also exhibit chemotherapeutic relevance. Since T is a substrate for TDG when mispaired with O^6-methylguanine [80, 81] (a cytotoxic lesion arising by using S_N1-type methylating chemotherapeutic agents) and O^6-methylguanine is a substrate for mismatch repair machinery when mispaired with either T or C, BER competes with MMR on the same substrate, thus maybe affecting the cytotoxicity of O^6-methylguanine-inducing drugs.

2.2.2.3 Methyl-CpG binding domain protein 4 (MBD4)

MBD4, also known as MED1, was identified by using two different screening methods. In 1998, Hendrich and Bird isolated the human and mouse MBD4 genes due to DNA sequence homology to the methyl-binding domain of MeCP2, a human 5-methylcytosine binding protein and transcriptional repressor [82]. The second identification of MBD4 was based on an interaction with the mismatch repair protein MLH1 that was found by using a yeast two-hybrid system [83]. The MBD4 protein is unusual in that it contains a methyl-CpG binding domain at the N-terminus [83] as well as a DNA glycosylase domain at the C-terminus [84-86]. Although structurally unrelated, MBD4 has enzymatic properties very similar to those of TDG and the optimal substrates are G/T and G/U mispairs in the context of CpG or 5-meCpG sites [85], suggesting a main role in counteracting the mutagenic potential of 5-meC deamination in CpG sequences. But other, albeit weaker, substrates for MBD4 have also been described: 5-meC from 5-meC/G [86], 5-FU from 5-FU/G [85] and T from T/O^6-meG [87] can all be removed by the action of this glycosylase.

Loss of MBD4 function in mouse embryonic fibroblasts causes several mismatch repair (MMR) proteins to be down-regulated, an effect manifested at the protein level but not the mRNA level [87]. This could be interesting, because it may show a functional link between the BER and MMR pathways.

The interaction discovered between MBD4 and FADD (Fas-associated death domain protein) may provide an explanation for the fact that MBD4-deficient cells fail to undergo apoptosis upon treatment with DNA damaging agents [87, 88].
MBD4-null mice are viable and fertile and are without apparent increase in tumor susceptibility, although they show a small increase in C → T mutations at CpG sites, which would be consistent with a defect in the repair of deaminated 5-meC that cannot be fully compensated for by the presence of TDG [89, 90]. On the other hand, the group of Primo Schär found that inactivation of TDG in mouse embryonic stem cells and fibroblasts reduces the G/T processing in cell extracts to undetectable levels, indicating that TDG provides the predominant activity against the products of 5-meC deamination in these cells (Christophe Kunz, Yusuke Saito, Primo Schär, manuscript in preparation).
The only conclusion that can be drawn at this point is that G/T repair in vertebrate cells is conducted by at least two distinct DNA glycosylases that may act in a partially redundant manner.

2.2.2.4 Single strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1)

In an attempt to sort rapidly through libraries of expressed proteins to detect DNA repair activity, Haushalter and colleagues devised an *in vitro* expression cloning (IVEC) approach and identified the SMUG1 protein from *Xenopus laevis*. Hence, the human counterpart was found due to extensive homology of the frog protein to several human expressed sequence tags (ESTs). Nicking assays revealed that both xSMUG1 and hSMUG1 acted upon U/A and U/G with no preference for either, but processed uracil residues in single-stranded DNA much more efficiently than in double-stranded DNA. Neither the *Xenopus* nor the human protein showed any enzymatic activity on G/T mismatches. hSMUG1 is furthermore localized to the nucleus [91], but is not cell cycle-regulated and, in contrast to UNG2, does not accumulate in replication foci [50]. This would agree with the suggested function of hSMUG1 being the main enzyme to eliminate U from U/G mismatches resulting from cytosine deamination [47]. Other studies, however, claim that UNG2 may be the key player in the repair of deaminated cytosines [50].

SMUG1 is not found in bacteria and yeast, but is present in higher eukaryotes. Boorstein *et al.* proposed hSMUG1 as the major enzyme for removal of 5-hydroxymethyluracil (5-hmeU) [92]. 5-hmeU is a result of oxidation of the 5-methyl group of thymine in DNA due to ionizing radiation and other forms of oxidative stress [93]. It is also the product of the deamination of 5-hydroxymethylcytosine, which may be formed via oxidation of 5-methylcytosine [94]. The latter forms a 5-hmeU/G base pair, which would be mutagenic if left unrepaired.

A recent study from Tomas Lindahl's lab attributes SMUG1 a possible role in cancer chemotherapy. Their data show that accumulation of 5-fluorouracil (5-FU) in the DNA of 5-FU-treated Smug1-deficient mouse embryonic fibroblast cells is cytotoxic and overexpression of Smug1 in wild-type cells protects cells against 5-FU-mediated killing [95]. From our *in vitro* studies we cannot confirm a significant contribution of hSMUG1 to the repair of 5-FU-containing mismatches (see ***Results***, Fischer *et al.*, manuscript submitted).

2.3 Mismatch repair (MMR)

2.3.1 Where it all started…

DNA damage has different faces, including strand breaks, a variety of chemical alterations of the standard bases, incorporation of cytotoxic base analogs, but also classical mispairs and insertion-deletion-loops (IDLs) that have escaped the proofreading exonuclease activity during DNA replication. The latter lesions become substrates for the mismatch repair system (MMR), whose task is to restore the information contained in the template strand. But to accomplish this challenging mission, the responsible repair system must be able a) to recognize base-base mismatches and IDLs, and b) to distinguish between the parent and the daughter strands [96]. Whereas other repair systems (e.g. BER) recognize modified or damaged DNA components, MMR addresses mismatches composed of unmodified standard nucleotides and therefore depends on a solid strand discrimination mechanism in order to avoid correction of the wrong moiety.

Due to his enormous experience with restriction enzymes and based on the finding that the transient undermethylation of the newly synthesized strand in *E. coli* might

provide the bias for strand discrimination [97, 98], Modrich and colleagues described in 1983 a substrate that permitted *in vitro* analysis of mismatch repair, thereby creating a breakthrough in this field. They could show that the *in vitro* repair activity in *E. coli* extracts is dependent on ATP, the state of *dam* methylation of mismatch heteroduplexes, and products of *mutH, mutL, mutS* and *uvrE* loci [99].
This provided the signal for a series of elucidating experiments and since the discovery of a link between the MMR system and hereditary non-polyposis colon cancer (HNPCC) in 1993 [100, 101], the study of this complex repair pathway proceeded in quantum leaps.

2.3.2 Biological significance of MMR

MMR corrects not only replication errors, but also mismatches that arise as a natural consequence of genetic recombination when the heteroduplex intermediate spans genetic differences between recombining helices [102, 103]. The absence of MMR permits illegitimate recombination between quasi-homologous sequences [104, 105]. In addition, a variety of base pair anomalies are also subject to processing by mismatch repair. These include base pairs containing O^6-methylguanine [106-110], 8-oxoguanine [111, 112], carcinogen adducts [113], UV photoproducts [114-116], and cisplatin adducts [107, 117, 118].
Genetic inactivation of the mismatch repair system elevates spontaneous mutability 50-1000-fold [119-124] and defects in this repair complex result in highly elevated rates of base substitutions and frameshift mutations, and render mammalian cells resistant to the cytotoxic effects of several classes of DNA damaging agents [123, 125-127]. The remarkable discovery of a causal relationship between deficiencies in MMR and a familiar form of human colorectal cancer (hereditary non-polyposis colon cancer, HNPCC) [128-131] and the inactivation of mismatch repair in a subset of sporadic tumors occurring in a variety of tissues [129, 131-134] make this repair pathway an important clinical target in the fight against cancer.

2.3.3 MMR in E. coli

The mechanistic steps of MMR are well defined in *E. coli* and the entire repair reaction has been reconstituted *in vitro* [135]. Biochemical and genetic studies

implicated eleven activities in methyl-directed mismatch repair, namely MutS, MutL and MutH proteins, DNA helicase II (also called UvrD), exonuclease I (ExoI), exonuclease VII (ExoVII), Rec J exonuclease, exonuclease X (ExoX), single-stranded DNA binding protein (SSB), DNA polymerase III holoenzyme and DNA ligase [135-138]. The repair reaction can be divided into three steps: initiation, excision, and resynthesis. In the initial phase, the homodimeric MutS [139, 140] protein detects a mismatch in a process that requires ATP and, upon interaction with a homodimer of MutL [139, 140] in the presence of ATP, activates the single-strand endonuclease MutH, which incises the unmethylated strand of a hemimethylated GATC site 5' to the G [141, 142]. MutH incision can occur either 3' or 5' to the mispair on the unmodified strand, providing MMR with a bidirectional capability. How the communication between MutS-MutL and MutH occurs is still a matter of debate. There exist three different models for ATPase function in MutS and MutS homologues at the moment, each with experimental support. In one model ("ATP-dependent translocation model"), on the basis of electron microscopic visualization, the MutS-MutL complex is proposed to loop out the DNA in an ATP hydrolysis-dependent manner, actively searching for the nearest d(GATC) methylation site either 5' or 3' to the mismatch [143-145]. A second model ("sliding clamp model") suggests that MutS-MutL or MutSα-MutLα bind the mismatch in an ADP-bound state, which provokes an ADP-ATP exchange. The ATP-bound complex undergoes a conformational change into a clamp with reduced affinity for the mismatch and diffuses freely along the DNA in an ATP hydrolysis-independent fashion. Thus, MutS-MutL or MutSα-MutLα complexes may interact with and activate the MutH endonuclease in the bacterial scenario [146] and may induce downstream events in eukaryotes [147, 148], respectively. The third model ("induced fit model") proposes that MutS (or MutSα) remains bound at or near the mismatch during the course of repair, thus challenging the idea that MutS (or MutSα) leaves the mismatch [149]. In this model, interaction of the mismatch and the strand signal is mediated by DNA bending. While details about this communication remain to be further elucidated, it is clear that once a nick at the nearest hemi-methylated d(GATC) site is generated, the MutS-MutL heteroduplex complex is also sufficient to activate the methyl-directed excision step, which includes DNA helicase II (UvrD) and several single-strand

specific exonucleases with appropriate polarity [136, 150]. By the action of these additional set of proteins, the DNA tract between the incised, hemi-methylated GATC site and the mismatch is removed [151, 152] and resection of the damaged DNA stops about 100 bp past the mismatch [143]. The single-stranded template is stabilized by single-stranded DNA binding protein (SSB) until DNA polymerase III holoenzyme is recruited to resynthesize the gap. In the last step of resynthesis, DNA ligase seals the nick, thereby completing the repair process and restoring the integrity of DNA [135, 140] (**Fig. 8**).

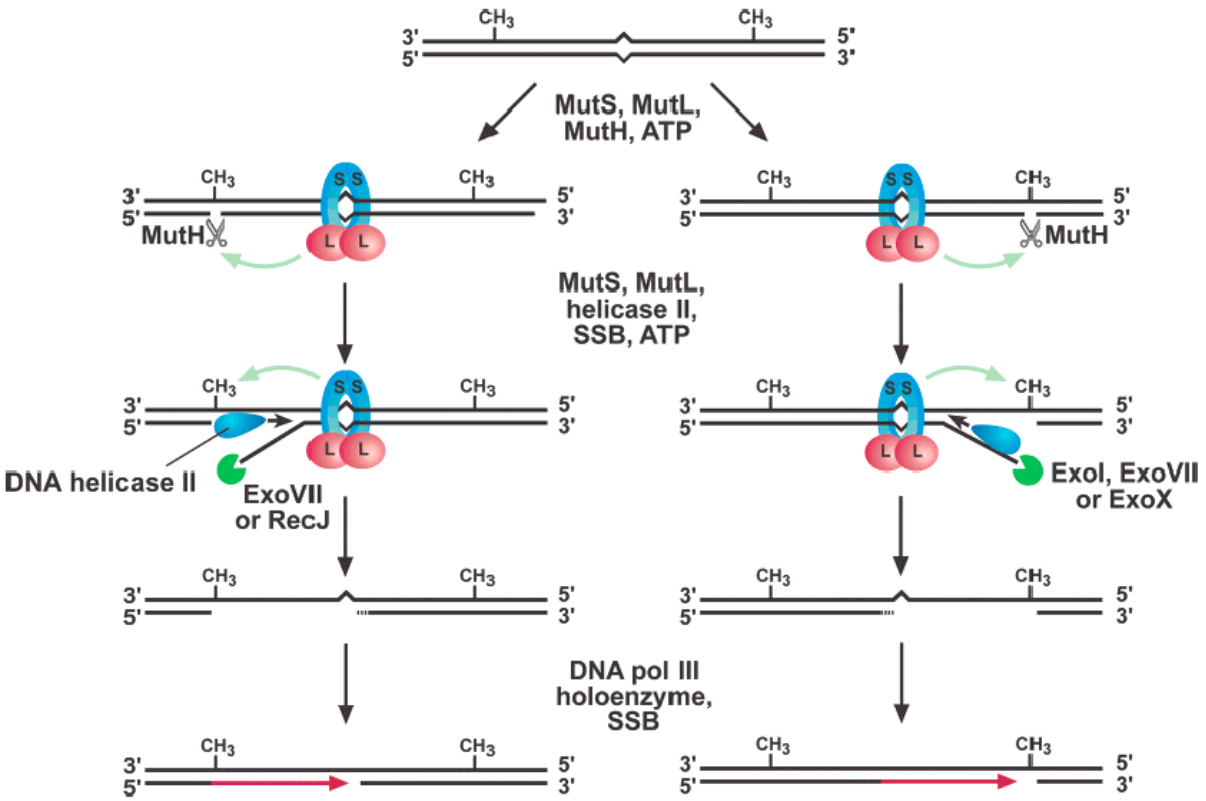

Figure 8 Mechanism of *E. coli* methyl-directed mismatch repair. Details of the reaction are described in the text. Although not shown, DNA ligase restores covalent continuity to the repaired strand after DNA polymerase III holoenzyme fills in the gap. Green arrows indicate MutS- and MutL-dependent signaling between the two DNA sites involved in the reaction [266].

2.3.3.1 E. coli MutS protein

MutS, a 95 kDa polypeptide, exists as an equilibrium mixture of dimers and tetramers [140, 153] and recognizes seven of eight possible mismatched base pairs (C/C is refractory to the bacterial protein) and binds to IDLs 1-4 nucleotides in length [140, 154-156]. The crystal structure of *E. coli* MutS bound to a G/T mismatch [157] revealed that the MutS homodimer encircles the mismatch-containing DNA as a pair of "praying hands" (reviewed in [158]), with the thumbs folded inwards, and the DNA passing between the fingertips and the thumbs (**Fig. 9**).

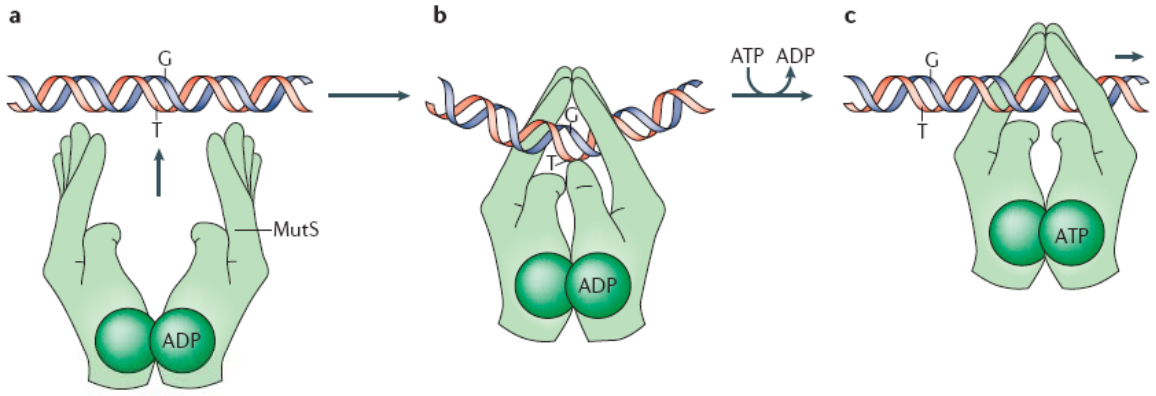

Figure 9 The MutS sliding clamp and its activation.
a The ADP-bound MutS homodimer binds to a G–T mismatch in duplex DNA. In the absence of DNA, the finger domains are unstructured and open, and the ATP-binding sites are dimerized.
b In the presence of the mismatched DNA, the ADP-bound form of MutS is wrapped around the DNA like a pair of praying hands, and is anchored at the mismatch site by a Phe-X-Glu wedge (the thumb of one of the hands) that is inserted into the minor groove of the duplex.
c ADP/ATP exchange brings about a conformational change that releases the Phe-X-Glu thumb from the mismatch site, but leaves the fingers closed around the duplex. The clamp is now free to translocate along the DNA in either direction (only one direction shown here). Although no eukaryotic MutSα structure is available, biochemical experiments indicate that these heterodimers function analogously to the bacterial proteins. The idea of the 'praying hands' was taken from [158].

Each subunit consists of five distinct domains:

Domain I ("mismatch binding domain") at the N-terminus forms the top segment of the thumb and harbours the highly conserved GXFY(E) motif for mismatch recognition. The phenylalanine residue (F) was shown to be essential for mismatch recognition in the *E. coli* MutS protein [159], in the Msh6p of *S. cerevisiae* [160], and in hMSH6 [161].

Domain II (“connector”) and **Domain III** (“core”) constitute the backbone of each subunit, or in visual words the second and third segment of the thumb, and are involved in transmitting allosteric information of bound DNA cofactors to the ATPase domain.

Domain IV (“DNA clamp”) is, like **Domain I**, involved in DNA binding and forms the fingers.

Domain V (“ATPase”) contains the highly conserved Walker A and B motifs found in many DNA repair proteins [162]. The ATPase domains of the two subunits of the MutS homodimer are intertwined, an arrangement that is likely to coordinate conformational changes of both monomers [149, 157, 163], changing the affinity for mismatch-containing substrates [164, 165].

Remarkably, only one subunit contacts the DNA at the mismatch site, rendering MutS a functional heterodimer, in analogy with the asymmetric yeast and human counterpart MSH2/MSH6 [160, 161].

2.3.3.2 E. coli MutL protein

The homodimeric MutL is a 68 kDa polypeptide and serves to interface mismatch recognition by MutS to activation of downstream activities. It is recruited to the heteroduplex in a MutS- and ATP-dependent manner [139]. On the basis of sequence and structural analyses, MutL belongs to the ATPases of the GHKL (gyrase/Hsp90/histidine-kinase/MutL) family [166], with a conserved nucleotide binding site at the N-terminus (aa1-335), a flexible and poorly conserved linker, and a much less well conserved dimerisation domain in the C-terminal region (aa439-615). The crystal structure of the N-terminal 349 amino acid residues revealed that binding of ATP or the non-hydrolysable ATP analogue AMP-PNP brings about a dramatic structural change and dimerisation [167, 168]. The idea that ATP is used to modulate conformational changes in the protein, thereby facilitating interactions with other protein and/or DNA partners [167, 169], is supported by mutational studies showing that impairment of either ATP binding or ATP hydrolysis by MutS or MutL abolishes the mismatch repair process [170]. A physical interaction between the N-terminal ATPase domain of MutL and MutH has been detected by protein cross-linking [168, 171] and several groups suggest direct interactions of MutL with UvrD

[169, 172, 173]. One recent study describes the structure of the C-terminal dimerisation domain of bacterial MutL and the authors propose a working hypothesis for MutL-mediated mismatch repair in *E. coli*, in which a large central cavity might form around DNA upon ATP binding-dependent association of the N-terminal ATPase domains of the two MutL subunits dimerised at the C-termini [173]. Whereas no DNA binding but binding and hydrolysis of ATP by MutL is required for mismatch-dependent activation of MutH [170], they postulate that during activation of UvrD helicase, MutL contacts DNA adjacent to the mismatch site as well as where UvrD unwinds the duplex, thereby looping out the intervening sequence in its large central cavity. However, the role of ATP hydrolysis in UvrD activation *in vivo* remains speculative. Moreover, the above study conflicts with a revised model derived from a series of bioinformatic analyses of the quaternary structure of the C-terminal domain of MutL, suggesting a conserved hydrophobic surface patch as the responsible region for dimer formation [174].

Another enigmatic aspect of the MutL protein is the biological significance of its DNA binding activity in MMR. Several groups have shown that MutL binds both double- and single-stranded DNA with no sequence specificity [167, 170, 173, 175, 176], while others report that MutL does not bind DNA [177], or suggest that binding to DNA may be irrelevant to its function [146]. In addition, purified MutL catalyzes a very slow ATP hydrolysis reaction that is stimulated by the presence of ssDNA and is essential for MMR [167-169]. Recently, presented data from genetic and biochemical assays using a MutL protein containing a single point mutation at the conserved position 266 (MutL-R266E) and therefore exhibiting a DNA binding defect [167, 178] strongly suggest DNA binding as an indispensable part of the MMR process [179].

Given the multiple roles in MMR, MutL is likely to be the master coordinator in the mismatch repair reaction, with still a lot of secrets to disclose.

2.3.3.3 E. coli MutH protein

MutH is a monomeric endonuclease that cleaves DNA at hemi-methylated d(GATC) sites 5’ to the G [141, 142] and its activity is greatly stimulated in a mismatch-dependent manner by MutS, MutL and ATP. The crystal structure of *E. coli* MutH revealed a clamp-like structure with two “arms” separated by a large cleft that can

accommodate a DNA duplex [180]. When MutH incision occurs 5' to the mismatch, excision depends on ExoVII or RecJ exonuclease [137, 143], both hydrolyzing single-stranded DNA with 5' to 3' polarity [181, 182]. On the other hand, cleavage 3' to the mispair requires ExoI, ExoVII or ExoX [135-138, 143], all of which support 3' to 5' hydrolysis of single-stranded DNA [181, 183, 184] (**Fig. 8**).

Although the MMR system is highly conserved through evolution, the MutH function is not present outside of gram-negative bacteria. Organisms lacking MutH might initiate exonucleolytic degradation of the heteroduplex at pre-existing strand interruptions [185-187].

2.3.3.4 E.coli UvrD helicase

UvrD is a superfamily I DNA helicase that exhibits modest processivity (40-45bp) [188-190]. Since MMR can require unwinding of up to 1-2kb of DNA, MutL is proposed to load multiple molecules of UvrD in an iterative process onto the substrate to increase the rate of progressive unwinding and to facilitate the unwinding of long duplex regions (reviewed in [191]).

2.3.4 MMR in yeast

The genetically best-characterized eukaryotic MMR system is that of the yeast *Saccharomyces cerevisiae*. Six MutS (MSH1-MSH6) and four MutL (MLH1-MLH3 and PMS1) homologs exert diverse functions (**Table 1**): MSH1 is required for the repair and maintenance of mitochondrial DNA [103] and has not been identified in mammalian cells; MSH2, MSH3 and MSH6 are required for the stability of nuclear DNA [192, 193]; and MSH4 and MSH5 are involved in meiotic recombination processes [194, 195]. The mitochondrial-specific function of MSH1 suggests that a primitive version of this protein may be the founding MutS family member in eukaryotic organisms, and recent phylogenetic analysis supports this hypothesis [196].

The current model of yeast MMR is that mismatch recognition is effected by two distinct MutS-like heterodimers composed of MSH2 together with either MSH3 or MSH6. *In vitro* binding studies have demonstrated that MSH2-MSH6 binds to base-

base mismatches or insertion/deletion loops, whereas MSH2-MSH3 binds only to duplexes containing insertion/deletion loops [197-200].

Like the MutS protein in *E. coli*, the yeast MSH proteins possess ATP binding and hydrolysis activity that is located in the highly conserved C-terminal region. In the presence of ATP, MSH2-MSH6 mismatch binding is abolished *in vitro* and results in proteolysis-sensitive conformational changes in the complex [201-203], presumably reflecting the sliding clamp feature of MutS proteins (**Fig. 9**).

The first evidence of a close conservation of the MMR pathway was the identification of the yeast MutL homolog PMS1 (yeast PMS1 = human PMS2), whose name reflects the fact that *pms1* mutants exhibit increased levels of postmeiotic segregation [204, 205]. The remaining three MutL homologs (MLH1-MLH3) were identified on the basis of amino acid conservation with MutL [206, 207]. MLH1 forms heterodimers with the remaining three MutL homologs [208]. The MLH1-PMS1 heterodimer is the major player in MMR [207], whereas MLH1-MLH2 and MLH1-MLH3 complexes are specialized to repair distinct classes of mutational intermediates [206, 209].

	E. coli		*S. cerevisiae*	
Initiation	MutS	Binds to mismatches and small IDLs	Msh2/Msh6	Binds to mismatches and 1 base IDLs
			Msh2/Msh3	Binds to IDLs. Has a role in double strand break repair
			Msh4/Msh5	No role in repair. Meiosis-specific MutS homolog
	MutL	Molecular matchmaker. Interacts with MutS in a mismatch-specific manner to activate MutH cleavage activity	Mlh1/Pms1	Primary MutL homolog for post-replication repair
			Mlh1/Mlh3	Involved in repair of some IDLs; also functions in meiosis
			Mlh1/Mlh2	Minor role in suppression of frameshifts
	MutH	Nicks unmethylated strand at a hemimethylated GATC site, initiating repair		No known homolog
	β-clamp	Processivity clamp for Pol III. Interacts with MutS in vitro. May recruit MutS to the replication fork	PCNA	Processivity clamp for pol δ and ε. Interacts with Msh3 and Msh6, increases mispair binding specificity and possibly delivers MutS to newly synthesized DNA. Also involved in repair resynthesis.
Excision	Helicase II	(UvrD) delivered to nick by MutS and MutL. Unwinds DNA prior to its excision		No known homolog
	RecJ	Required for 3'- 5' excision between nick and mismatch	ExoI	5'- 3' exonuclease, deletion of which results in a mild mutator phenotype
	ExoVII			
	ExoI	Required for 5'- 3' excision between nick and mismatch		
	ExoX			
Resynthesis	pol III	DNA polymerase required for repair resynthesis	pol δ	DNA polymerase required for repair resynthesis
	SSB	Single strand DNA binding protein. Aids excision and resynthesis	RPA	Single strand DNA binding protein. Aids resynthesis
	ligase	Seals nicks	ligase	Seals nicks

Table 1 *E. coli* and *S. cerevisiae* proteins required for DNA mismatch repair (from [124]).

MSH4 and MSH5 were identified because of their effects on meiotic recombination, and show no detectable involvement in mismatch correction [194, 195], consistent with the absence of a conserved N-terminal MutS/MSH domain responsible for mismatch binding [196].

The availability of yeast MMR mutants were of inestimable value for the understanding of MMR-deficient human cell phenotypes and the identification of human MMR genes [210-214], thanks to the conservation of the amino acid sequences from bacteria to yeast to man.

2.3.5 MMR in higher eukaryotes

2.3.5.1 The leading actors

Although eukaryotic cells possess a MMR pathway that is homologous to the *E. coli* methyl-directed, MutHLS-dependent repair [186, 187], there is a major difference between the two systems: While the bacterial MutS and MutL proteins are homodimers, the eukaryotic counterparts function as heterodimeric complexes. Among the several eukaryotic homologues of MutS (designated MutS homologues MSH2 to MSH6), three are involved in mitotic genetic stability where they participate in repair of base-base mismatches and IDLs: MSH2 interacts with MSH6 or MSH3 to form MutSα [215] or MutSβ [216, 217], respectively. MutSα supports repair of all eight base-base mismatches including C-C, as well as ID mispairs containing up to about ten unpaired nucleotides, whereas MutSβ corrects IDLs containing two to about ten nucleotides but is only weakly active on single nucleotide mismatches [216, 217]. MSH4 and MSH5 – as shown for their yeast counterparts - are restricted to meiosis where they play important roles in crossing over [218, 219].

Albeit not as well studied as the MutS homologues, four MutL counterparts, MLH1, MLH3, PMS1 and PMS2, have been identified [213, 214, 220, 221]. MutLα, a MLH1-PMS2 heterodimer [222, 223], is capable of supporting repair initiated by MutSα or MutSβ [215], therefore playing the first fiddle in MMR. MutLβ, which is composed of MLH1 and PMS1, could not be shown to participate in MMR *in vitro* [224]. Nevertheless, it cannot be completely ruled out that this heterodimer might be involved to some extent in some mismatch repair events, since *Pms1*-knockout mice

exhibit low microsatellite instability (MSI) in mononucleotide runs [225]. Interestingly, latest findings from the lab of Paul Modrich identified an endonuclease activity in the subunit PMS2 of MutLα, with the consensus endonuclease sequence being conserved in MLH3, but not in PMS1. This explains the observation that MutLβ shows no or only marginal MMR activity [226]. Similarly, MutLγ, consisting of MLH1 and MLH3, which appears to be involved in meiosis [227], participates, albeit inefficiently, in the repair of base-base mismatches and single-nucleotide IDLs ([228], see ***Results***, Cannavo *et al*. (2005), Cancer Research).

In addition to MutS and MutL homologues, human mismatch repair requires several other factors, one of which is the homotrimeric proliferating cell nuclear antigen (PCNA). By interacting with MSH3 and MSH6 [229-231], PCNA has been shown to be of importance not only during DNA resynthesis as a processivity factor for polymerase δ [232], but already during MMR initiation prior to the excision steps [233]. Thus, PCNA may exert multiple functions in the process of MMR, with proposed roles in facilitating mismatched DNA binding and mobilizing the MutSα/MutLα complex away from the mismatch [234], and suggested functions as a strand discrimination signal [233, 235] or as a key protein in the differential activation of the appropriate hydrolytic system [236].

Another group of important players are the exonucleases. Since the human MMR system acts bidirectionally, degradation in 3'→5' and 5'→3' must be accomplished. Yeast genetic studies implicated the 3'→5' proofreading activities of DNA polymerase δ and/or polymerase ε in MMR [237, 238], but no significant involvement of the polymerase δ editing exonuclease was evident in human mismatch-provoked excision *in vitro* [239]. Other findings indicated that the 3'→5' exonuclease activity of MRE11 might also be required *in vivo* [240]. Among the 5'→3' exonucleases, only exonuclease-1 (EXO1) has been shown to act *in vivo* and *in vitro* [241], in agreement with recent *in vitro* results, showing that EXO1 is sufficient for both 3'→5' and 5'→3' mismatch correction [236]. Nevertheless, it can be concluded from knock-out mice models that there must be some redundant activities besides EXO1 *in vivo* [242].

2.3.5.2 Human MutSα

In 1988, a first description of a factor binding G/T mispairs in HeLa extracts was published [243]. Analogous to its function, it was named GTBP (G/T-binding protein) and the purest fraction of this activity contained two bands of apparent molecular mass of 100 and 160 kDa [244], the smaller of them thought to be formed by proteolysis of the larger one. This hypothesis, however, turned out to be wrong: the observed bands represent two proteins encoded by distinct genes [245]. The 100 kDa protein was shown to be the product of the *hMSH2* gene [246] and the 160 kDa protein, originally called GTBP [244], was renamed hMSH6 due to its close relatedness to the *S. cerevisiae* MSH6 protein [193]. These results mirror the asymmetric nature of the hMutSα heterodimer and implied that the two subunits play distinct roles during mismatch recognition. Indeed, cross-link experiments with mismatch-containing DNA only succeeded with hMSH6 [164]. And correspondingly to the finding of Malkov *et al.* with MutS protein of *T. aquaticus* [159], a Phe→Ala substitution in the highly conserved N-terminal motif of hMSH6, but not hMSH2, severely attenuated binding and mismatch repair functions of hMutSα [161]. A similar effect could be shown by mutating the equivalent amino acid in the Msh6p of *S. cerevisiae* [160].

MutS homologues are members of the ABC (ATP binding cassette) transporter family of ATPases [247] with their C-terminal domains being highly conserved.

Since the Phe-X-Glu motif described above resides in the N-terminus and is therefore far away from the C-terminal ATP-binding sites, nucleotides are dispensable for the initial binding of MutSα and MutSβ to their respective substrates [215, 217, 245, 248]. This was confirmed by the findings that mutations in the ATP-binding sites of different MutS homologues had little effect on mismatch binding, but resulted in a significant decrease in the ATP-driven dissociation of the proteins from DNA [164, 201, 203, 249]. On the other hand, mutations in ATP-binding sites of either subunit of hMutSα, but particularly in hMSH6, resulted in MMR deficiency in an *in vitro* assay [164], as well as *in vivo* [165].

One major difference between hMutSα and MutS is the simultaneous occupancy of the nucleotide binding sites: whereas MutS sliding clamp contains two bound ATP molecules, hMutSα has two nucleotide binding sites with differential specificities for ADP and ATP [250].
However, the roles of nucleotides in MMR need to be further elucidated.

2.3.5.3 Human MutSβ

Repair assays with extracts from cells deficient either in MSH6 or MSH2 proteins revealed that although MSH2-deficient extracts lacked the ability to repair G/T mismatches and IDLs, MSH6-deficient extracts were partially proficient in the repair of two-nucleotide loops [215], implying the existence of a MSH6-independent loop repair.
Subsequently, Palombo *et al.* showed that MSH2 forms by heterodimerization with MSH3, MutSβ [217]. MutSβ is responsible for the repair of IDLs in the range of two to ten nucleotides, weakly recognizes single-nucleotide IDLs and is essentially inert on base-base mismatches [216, 245]. *In vivo*, the ratio of MutSα/MutSβ is around ten to one [216, 251, 252]. Overexpression of MSH3 leads to a dramatic change in the relative levels of MutSα and MutSβ, with the consequence of base-base mismatch repair deficiency due to depletion of MutSα [253].

2.3.5.4 Human MutLα

The human counterpart of the bacterial MutL is a heterodimeric factor, MutLα, consisting of MLH1 and PMS2 [222], and although it is essential for MMR, its precise function is unclear. MutLα accounts for about 90% of the MLH1 in human cells [224, 228], the remaining MLH1 is involved in the formation of two low abundance complexes, MutLβ (MLH1/PMS1; [224]) and MutLγ (MLH1/MLH3; [221]).
In the presence of ATP, human MutSα and human MutLα form relatively stable ATP-dependent ternary complexes on oligonucleotide substrates [223, 254, 255]. Since substitutions in the ATP binding sites of MutLα do not affect the formation of the ternary complex with MutSα on DNA, but induce deleterious effects, the latter could

be linked to a step downstream [223], confirming the reputation of MutL proteins as so-called “molecular matchmakers”.

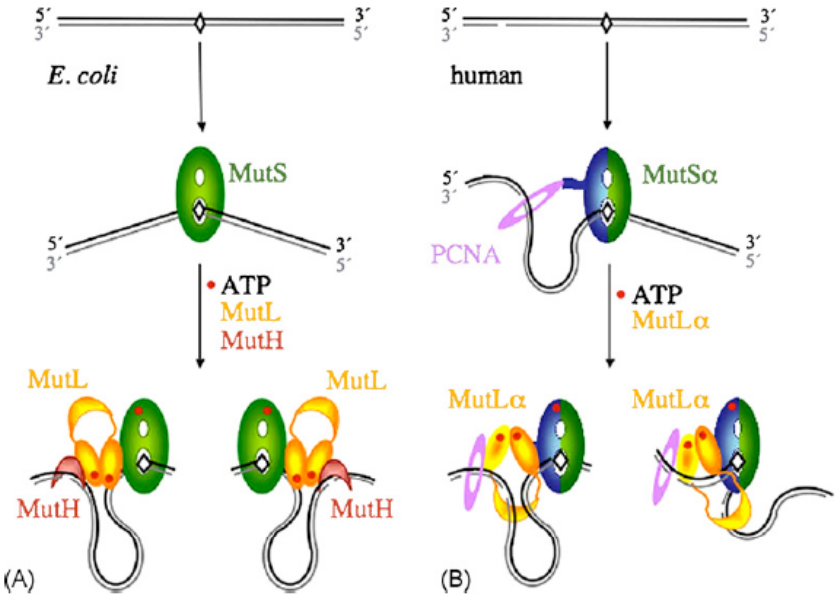

Figure 10 Diagrams of the initial incision step of MMR in *E. coli* and humans.
A In *E. coli*, MutS recognizes a mismatch, binds it and bends the DNA by 60° towards the major groove. The newly synthesized and unmethylated daughter strand is shown in grey. In the presence of ATP, MutL is recruited to the MutS-mismatch complex, and together they activate MutH to nick the daughter strand on either the 5’ or 3’ side of the mismatch. Since the DNA-binding activity of MutL is not necessary for this step and slightly inhibits MutH activation [170], we propose that the DNA in between the mismatch and incision site is looped out.
B In humans, MutS is made of MSH2 (green) and MSH6 (blue) and interacts with PCNA (purple). The daughter strand is marked by a pre-existing strand break. A break 3’ to the mismatch is shown here as an example. MutS may have a strand preference when loaded by PCNA. The ensuing MutS–MutL-mismatch complex could also be biased due to the asymmetric (heterodimeric) nature. Incisions by the PMS2 subunit of MutL on the 3’ side of the mismatch may be guided by PCNA and MutS, and incisions on the 5’ side may be limited by how far the C-terminal domain of MutL (the endonuclease active site) can extend from the MutS–MutL complex located on the mismatch site (taken from [256]).

Recently, Paul Modrich and his colleagues could celebrate another triumph in the MMR field by the discovery of an intrinsic endonuclease activity in the PMS2 subunit of MutLα [226]. After having speculated for a long time about an unknown cryptic 3’→5’ exonucleolytic activity, the authors observed cleavage of the daughter (discontinuous) strand on either side of the mismatched base, guiding them finally towards the answer to this problem (**Fig. 10**).

2.3.5.5 Human MutLβ

PMS1 is expressed in human cells and interacts with MLH1 with high affinity to form the heterodimer MutLβ [224]. Since tumor cell lines lacking either MLH1 or PMS2 exhibit apparently similar phenotypes [257], a significant functional redundancy

between PMS2 and other MutL homologues was excluded. In addition, mice carrying a disruption of the Pms1 gene display neither MSI nor cancer predisposition [225]. However, identification of one patient with an HNPCC family background who harbored a mutation in the PMS1 gene [213] and the findings that the PMS1 homologue in yeast, Mlh3p, interacts with Mlh1p in a yeast two-hybrid assay and contributes to the repair of a subset of IDLs [122, 206, 258], challenged this statement. Nevertheless and although biochemically characterized, participation of MutLβ in mismatch repair could not be shown so far and thus remains to be demonstrated.

2.3.5.6 Human MutLγ

Human MutLγ, a heterodimer consisting of MLH1 and MLH3, was identified by Far Western Blots [221], but its role in MMR was not characterized. Studies in *S. cerevisiae*, where MLH3 was first identified, showed an interaction between scMlh3p and scMlh1p [206, 208]. Furthermore, involvement of MLH3 in yeast [259, 260], human [261, 262] and murine [262] meiotic recombination has been reported. Recently, we could confirm the existence of hMutLγ *in vivo* and suggest a back-up role in MMR for this heterodimer in correcting base-base mispairs and one nucleotide IDLs with low efficiency [228].

2.3.5.7 Reconstituted MMR systems

Based on data deriving from reconstituted *in vitro* systems [239, 263], the current scheme of human MMR suggests that the mismatch-bound MutSα undergoes an ATP-dependent conformational change, converting the heterodimer into a sliding clamp which is capable of translocating along the DNA backbone *in vitro* [145, 148, 165].

In a second ATP-dependent step, the MutSα-ATP-DNA complex recruits MutLα [222], forming a mobile complex with the ability to translocate bidirectionally along the DNA contour in search of a strand discontinuity. Although endowed with bidirectionality, different protein sets are required, depending on the polarity of the encountered nick.

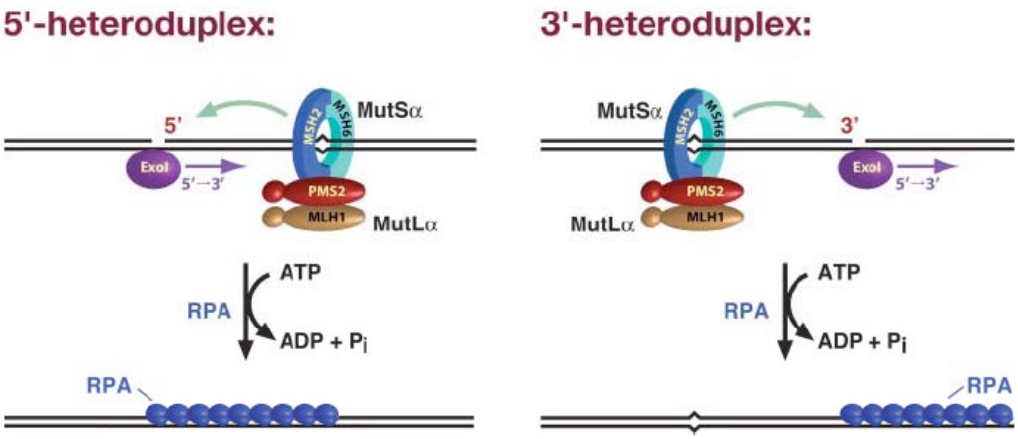

Figure 11 5' to 3' default hydrolytic system. EXO1, which is activated in a mismatch- and MutSα dependent manner, initiates 5' to 3' hydrolysis at the strand break [263-265]. Excision on a 5'-heteroduplex terminates upon mismatch removal in a manner that depends on RPA and MutLα. MutSα also activates EXO1 on a 3'-heteroduplex, but in this case hydrolysis proceeds with the wrong directionality for mismatch removal. *Green arrows* indicate a requirement for signaling between the mismatch, which activates excision, and the strand break where hydrolysis initiates (from [266]).

A four protein system, consisting of purified MutSα, MutLα, RPA and exonuclease-1 (EXO1), was shown to be sufficient to mediate excision of a 5'-heteroduplex (nick located 5' to the mismatch, **Fig. 11**), terminating in the region of 60 to 230 nucleotides beyond the mispair [265]. In this scenario, RPA enhanced MutSα-dependent EXO1 activation in the presence of MutLα. Even though MutLα is not essential for 5'→3' degradation, it improved the selectivity of the reaction in favor of the heteroduplex substrates.

Interestingly, exactly the same happened in the presence of a 3'-heteroduplex (nick located 3' to the mismatch, **Fig. 11**), resulting in an excision reaction proceeding away from the mismatch with incorrect 5'→3' polarity ([236], **Fig. 11**).

Supplementation of both PCNA and RFC (clamp loader of PCNA [267]) yields a system that supports mismatch removal from 5'- and 3'-heteroduplexes [236]. More precisely, MutSα, RFC, and PCNA activate a latent MutLα endonuclease in an ATP- and mismatch-dependent manner [226], which incises both 3'- and 5'-heteroduplexes with a strong bias for the nicked strand (**Fig. 12**).

Like this, a 5’ initiation start point on a 3’-heteroduplex is generated, subsequently leading to the removal of the mismatch by the 5’→3’ action of MutSα-activated EXO1 (**Fig. 12**).

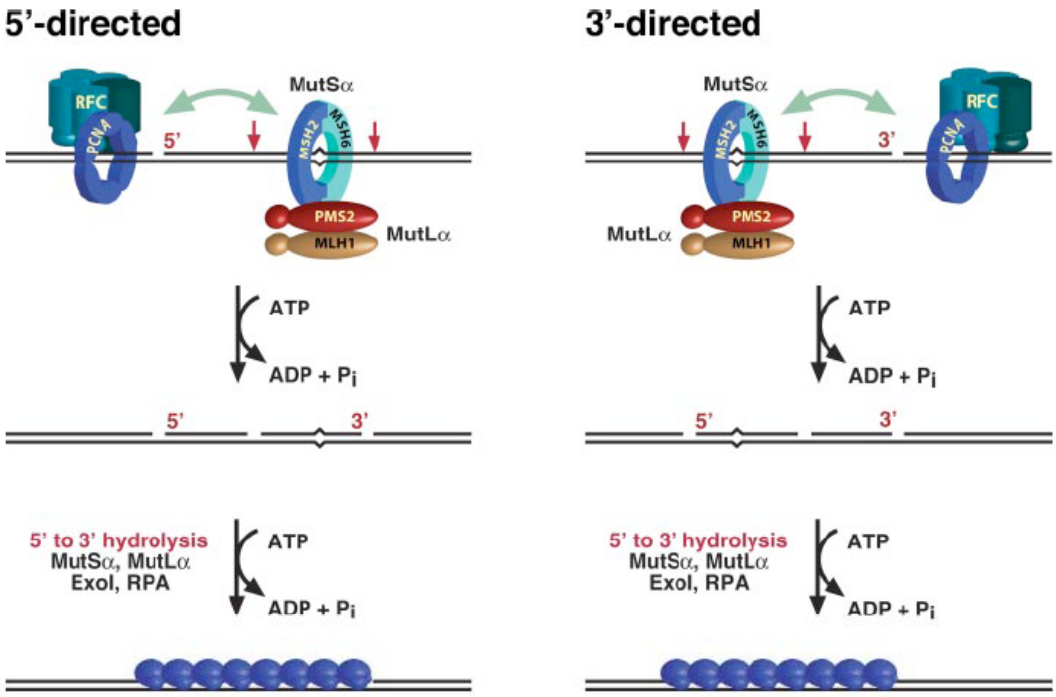

Figure 12 MutLα endonuclease in mismatch-provoked excision. MutLα endonuclease, which is activated in a mismatch-, MutSα-, PCNA-, RFC-, and ATP-dependent manner, incises (*red arrows*) the discontinuous strand of 5’- or 3’-heteroduplex DNAs in an ATP-dependent manner [226]. Incision of a 3’-heteroduplex on the distal side of the mispair relative to the strand break yields a 5’ terminus that serves as an entry site for MutSα-activated EXO1, which removes the mismatch by the 5’ to 3’ hydrolytic reaction shown in **Fig. 11**. Excision tracts shown in this model span the mismatch and the original heteroduplex strand break, as observed in human cell extracts [185, 187, 268]. Because MutLα activation depends on a heteroduplex strand break and because endonuclease action is restricted to the nicked heteroduplex strand, signaling must occur between mismatch and heteroduplex strand break (*green arrows*). Adapted from Kadyrov *et al.* [226].

By supplementing this six-component excision team with DNA polymerase δ and DNA ligase I, the MMR process could be fully reconstituted *in vitro* ([239, 263], **Fig. 13**).

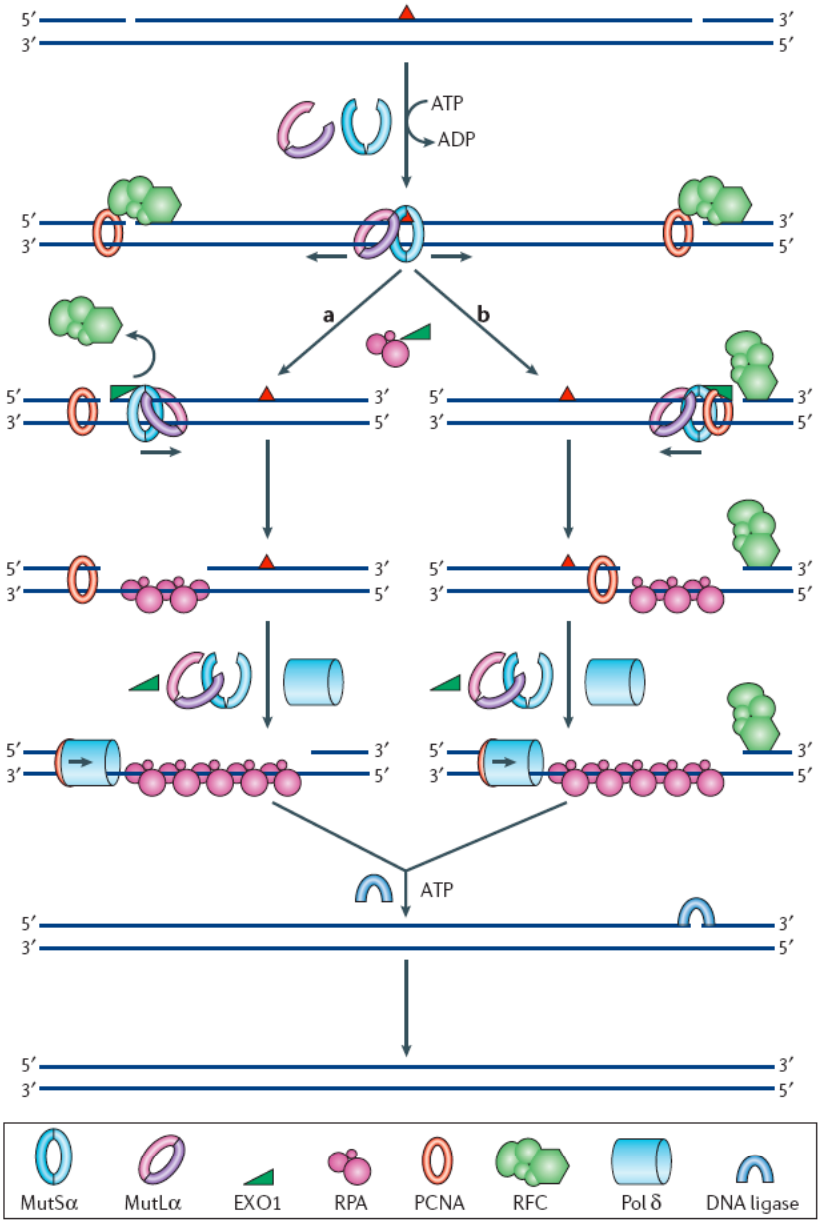

Figure 13 The reconstituted human mismatch-repair system. The mismatch repair (MMR) process was recently reconstituted [239, 263] from either MutSα or MutSβ, MutLα, replication protein A (RPA), exonuclease-1 (EXO1), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), DNA polymerase δ (Pol δ) and DNA ligase I. The following is proposed to take place. The mismatch (red triangle)-bound MutSα (or MutSβ) recruits MutLα. The ternary complex undergoes an ATP-driven conformational switch, which releases the sliding clamp from the mismatch site.
a Clamps that diffuse upstream encounter RFC that is bound at the 5' terminus of the strand break, and will displace it and load EXO1. The activated exonuclease commences the degradation of the strand in a 5'-3' direction. The single-stranded gap is stabilized by RPA. When the mismatch is removed, EXO1 activity is no longer stimulated by MutSα, and is actively inhibited by MutLα. Pol δ loads at the 3' terminus of the original discontinuity, which carries a bound PCNA molecule. This complex fills the gap and DNA ligase I seals the remaining nick to complete the repair process.
b Clamps that migrate downstream encounter a PCNA molecule that is bound at the 3' terminus of the strand break. The recruitment and the activation of EXO1 results in the degradation of the region between the original discontinuity and the mismatch, possibly through several iterative EXO1-loading events. RFC that is bound at the 5' terminus of the discontinuity prevents degradation in the 5'-3' direction (away from the mismatch). Once the mismatch is removed and the EXO1 activity is inhibited by bound RPA and MutLα, the gap is filled by Pol δ. DNA ligase I seals the remaining nick to complete the repair process (from [269]).

2.3.5.8 Other functions of MMR

Mammalian MMR is not only involved in correcting biosynthetic errors, but performs a wide choice of tasks in the field of DNA transactions. Here are some relevant and experimentally substantiated examples:

I) MMR and its role in homologous recombination

Mismatches arising as the result of heteroduplex formation during homologous recombination are processed by the same MMR machinery that removes DNA replication errors. Whenever MMR proteins encounter mismatch-containing recombination intermediates, two different processes can take place: simple mismatch correction or complete abortion of the recombinational event, thereby preventing strand exchange between similar but non-identical (homologous) sequences [104, 270]. Studies in *E. coli* demonstrated that inactivation of MutS and MutL dramatically increases the frequency of homologous exchanges between quasi-homologous sequences [271-274]. Subsequent studies with yeast and mammalian cells suggested similar functions for the MutS and MutL homologues [104, 105].

II) MMR and its role in meiosis

Initial studies of the involvement of MMR proteins in meiotic recombination were conducted in yeast. Mutants defective in MLH1 or MLH3 [275, 276] and in MSH4 and MSH5 [194, 195] show a two- to threefold reduction in crossing-over, thus indicating that the heterodimers MSH4-MSH5 and MLH1-MLH3 function during meiosis [208]. It has been hypothesized that MSH4-MSH5 and MLH1-MLH3 complexes promote the formation and stabilization of Holliday junctions and their preferential resolution into cross-overs rather than noncross-overs [277]. Interestingly, abnormal meiosis activates a checkpoint leading to cell death via Msh4-Msh5 and Mlh1-Mlh3 complexes [277], underscoring the ability of MMR proteins to signal DNA damage and promoting apoptosis (reviewed in [269, 278, 279]).

III) MMR and its role in triplet repeat instability

The cause of several neurogenerative diseases such as myotonic dystrophy, Huntington’s disease, fragile-X syndrome, and Friedreich’s ataxia is aberrant

expansion and hence instability of triplet-repeat sequences. MutSβ seems to play a major role in triplet-repeat destabilization, since repeat tracts are more stable in *MSH3*-null mice compared to *MSH6*-null animals [280]. Triple-repeat sequences readily form hairpins and mismatches in the context of these secondary structures cause aberrant recognition by Msh2-Msh3. In addition, altered nucleotide affinity and suppressed ATPase activity led to the assumption that the compromised function of MutSβ may lead to stabilization of the loops and consequently to expansion instead of repair [281]. But, despite all the existing models, the detailed molecular events remain to be established.

IV) MMR and somatic hypermutation of immunoglobulin genes

Antibody diversity is achieved by three different processes that take place in immunoglobulin genes during clonal expansion of B-lymphocytes: V(D)J recombination, class-switch recombination (CSR) and somatic hypermutation (SHM) [282]. The finding that MSH2-deficient mice showed diminished proportion of dA:dT mutations led to the model that somatic hypermutation acts in two phases:

The first stage requires activation-induced cytidine deaminase (AID), which converts C residues to U [283]. The consequently formed dU:dG mismatches then become substrate for a number of different DNA repair pathways. First, they may be fixed as transition mutations by replication. Second, UNG excises the uracil residue, thereby creating an abasic site. Should the latter become a template for replication, almost any other base can be incorporated opposite the empty position, resulting in transversion and transition mutations [284]. Last but not least, the dU:dG or the abasic site:dG are recognized by MutSα [116, 285], and by the possible involvement of DNA polymerases with low fidelity, mutations in the vicinity of the original dC deamination target can be introduced. Indeed, the interaction between MutSα and polymerase η stimulates synthesis of second stage mutations at bases located downstream of the initial dU lesion, including A:T pairs ([285], see below).

In a second stage, mutations are predominantly generated in dA:dT pairs. Deficiency in either MSH2, MSH6, EXO1 or polymerase η reduces mutations at dA:dT, but does not abolish them [286-295], thus indicating that MMR is only part of the process and not the sole path in phase two mutations.
It has also been suggested that MMR is an important part of the mechanism for conversion of single-strand breaks to double-strand breaks in class-switch recombination (CSR) [296].

3. The role of MMR in cancer and cancer therapy

3.1 MMR and HNPCC (hereditary non-polyposis colorectal cancer)

In attempts to unravel the mystery of the observed instability of microsatellites in 12% of the studied colorectal cancer (CRC) tissues [297, 298] and in an effort to locate the HNPCC gene(s) in families with early-onset CRC [100, 101], several groups of scientists, in 1993, drew nearer a nameless culprit. The markers used in the search for the HNPCC locus mapped to the short arm of chromosome 2 [100] and since they hybridized with microsatellite sequences [101], the suspicion arose that the candidate gene may encode a factor involved in DNA replication or repair. And, once again, mutator mutants of *S. cerevisiae* offered good services and provided the “missing link” by demonstrating convincingly that stability of simple repeats in mutator mutants lacking MMR was up to three orders of magnitude lower than in wild-type strains [299]. This was the starting signal for the identification of human MMR genes and their genetic loci.
Now we know that the cause of the familial syndrome HNPCC (also known as Lynch syndrome) is the inheritance of a mutated allele of a MMR gene and a concomitant predisposition to cancers of the colon and endometrium and, with much lower frequency, to cancers of the stomach, bladder, brain, ovaries and other organs. The reason underlying this predominance for cancer development in the colon is not known, but since the formation of adenomatous polyps in HNPCC patients arises about two decades earlier than in healthy individuals, the presence of a germline MMR mutation seems to increase the transformation rate in the colonic epithelium [300-302]. HNPCC is inherited in an autosomal dominant way, but MMR deficiency

only arises when the wild-type MMR allele is mutated or lost. Thus, the disease is recessive at the somatic level.

Colorectal cancers can be divided into two main groups according to their type of genetic instability [303-305]. Microsatellite instability, the hallmark of MMR deficiency, occurs in approximately 15% of all colorectal cancers and can arise through either inheritance of germline mutations in MMR gene(s) (5%) or epigenetic biallelic methylation of the MLH1 promoter (10%). In HNPCC, MLH1 and MSH2 alterations account for almost 90% of all identified mutations, MSH6 is mutated in about 10% of all cases and the share of PMS2 is less than 5% [134]. Detection of sequence variants in MLH3 in the germline of families predisposed to colorectal cancer [306, 307] suggests a possible role in human cancer, maybe in combination with other factors [228].

3.2 MMR, DNA-damage tolerance and clinical implications

DNA mismatch repair defects are common in a variety of cancers and can be detected by MSI analysis and/or immunohistochemical staining for MMR protein expression [308]. The major obstacle for the successful treatment of cancer is the resistance to chemotherapeutic agents. Interestingly, MMR-deficient cell lines are more resistant to killing by several different cytotoxic drugs than matched proficient ones (reviewed in [127]), making the MMR system an important indicator for cancer therapy and manifesting its involvement in DNA damage response [269, 279].

3.2.1 Methylating agents

Methylating agents are capable of reacting with a number of nucleophilic sites on DNA (**Fig. 14**), and can be subdivided into two families: S_N1 and S_N2 [309].

Methyl methane sulfonate (MMS) and dimethyl sulfate (DMS) are examples for S_N2-type (bimolecular nucleophilic substitution) agents, whereas *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU) and the chemotherapeutics temozolomide (TMZ) and dacarbazine [310] belong to the S_N1-type reagents. The predominant DNA adducts resulting from S_N1-type methylating agents are N^7-methylguanine, N^3-methyladenine, O^4-methylthymine, O^6-methylguanine and methyl-phosphotriesters.

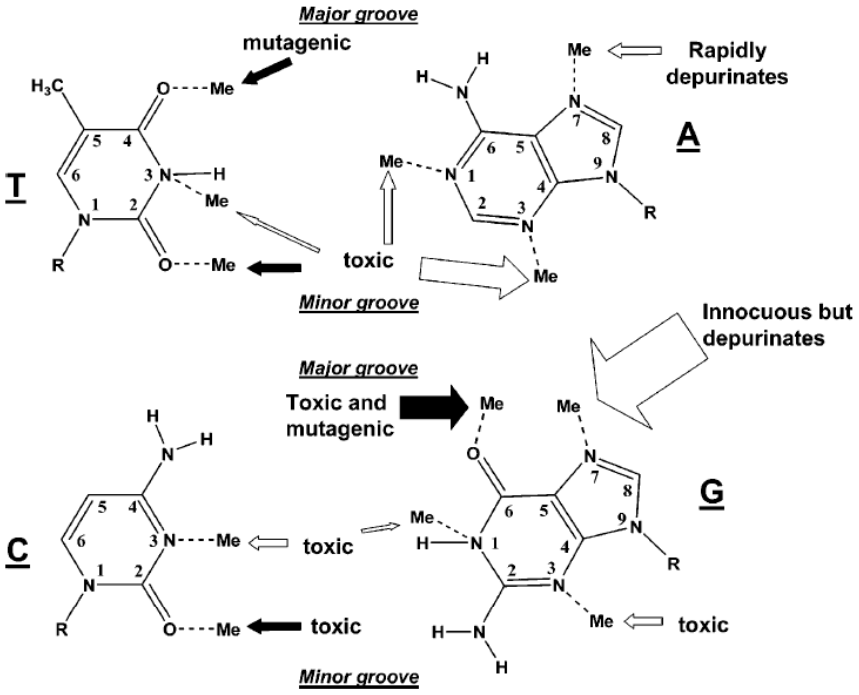

Figure 14 Potential sites of chemical methylation in double-strand DNA. The arrows point to each methyl adduct and whether the adduct is known to be predominantly toxic or mutagenic. The open arrows represent sites that are methylated by MMS, MNNG, and MNU. The filled arrows point to sites that are methylated by MNNG and MNU but not detectably by MMS. Note that the methylation of different sites on the same base at the same time is extremely rare. The size of the arrows roughly represents the relative proportion of adducts. In single-stranded DNA, the *N*1-adenine and *N*3-cytosine positions display a greater reactivity (from [309]).

N^7-methylguanine and N^3-methyladenine comprise ~90% of the damage [311] and can be efficiently removed by BER [25]. Interestingly, the cytotoxicity of S_N1-type reagents has been ascribed to O^6-methylguanine that accounts for only 7% of the adduct burden in DNA [311]. O^6-methylguanine can be repaired directly by O^6-methylguanine-DNA methyltransferase (MGMT, see **Direct Repair**) [12] and high levels of this enzyme protected cells against killing by S_N1-type methylating agents [125], which implicated O^6-methylguanine in this process. In addition, cell lines defective in both MMR and MGMT are generally ~100-fold more resistant to killing induced by S_N1-type methylating agents than matched proficient ones [125], providing evidence that the MMR system potentiates the toxicity of DNA damaging drugs such as MNU and MNNG. But the mechanism of MMR-potentiated cell killing due to O^6-methylguanine lesions remains enigmatic, although a number of models, which will be discussed, have been proposed.

The “futile repair” hypothesis has been discussed already in 1982 by Karran and Marinus [108] and suggests that the MMR system, which is exclusively directed to

the newly synthesized strand, encounters O^6-methylguanine in the template. Unsuccessful attempts to process the lesion lead to iterative degradation and re-synthesis cycles, ultimately resulting in secondary DNA intermediates, such as gaps. The persistence of these aberrant DNA structures into the second cell cycle may trigger cell killing due to harmful recombination intermediates [312]. The idea of such a scenario is not new: in 1971, Plant and Roberts suggested that replication past O^6-methylguanine may produce single-stranded gaps that are converted to double-strand breaks in the second cell cycle [313]. Interestingly, MMR-proficient cell extracts perform aberrant O^6-methylguanine-dependent DNA synthesis and inhibit DNA replication on *in vitro* methylated substrates, a phenomenon which cannot be observed in MMR-deficient extracts, thus representing a first biochemical proof for a direct link between MMR and O^6-methylguanine-derived cytotoxicity [314-316]. Recent findings from our lab perfectly confirm this model by showing that MNNG treatment induced a MMR-dependent cell cycle arrest only in the second G2 phase after treatment [312]. MMR-dependent formation of single-stranded regions behind yeast and mammalian replication forks upon MNNG treatment could be visualized by electron microscopy and may further substantiate the above-described hypothesis (preliminary unpublished data from Massimo Lopes). In summer 2006, York and Modrich supplied first evidence for the “futile repair” model *in vitro* by studying MMR-dependent iterative excision on heteroduplex substrates containing a single O^6-methylguanine lesion incubated with nuclear extracts [317]. Although testing different approaches we could not confirm the futile cycling scenario on our heteroduplex substrates (see ***Results***, Fischer *et al*., manuscript in preparation).

An alternative to the “futile repair” model, the “sliding-clamp signaling” model, suggests that O^6-methylguanine is recognized by MutS homologues and the ATP-activated sliding clamps translocate along the DNA, thereby directly signaling apoptosis ([318], **Fig. 15**), without the need for DNA processing.

3.2.1.1 Temozolomide (TMZ) and dacarbazine

Dacarbazine is considered to be the most active drug for the treatment of metastatic melanoma, with a response rate of 15-20% [319]. As in the case of 5-FU, a number of combination regimens which include dacarbazine increase this response rate.

Temozolomide spontaneously decomposes into the active metabolite of dacarbazine [320] and recently has been approved for the treatment of recurrent gliomas. Furthermore, it is in phase II/III clinical trials for the treatment of melanoma. Since temozolomide penetrates the blood-brain barrier, it could be beneficial in preventing or treating melanoma metastases in the central nervous system [319].

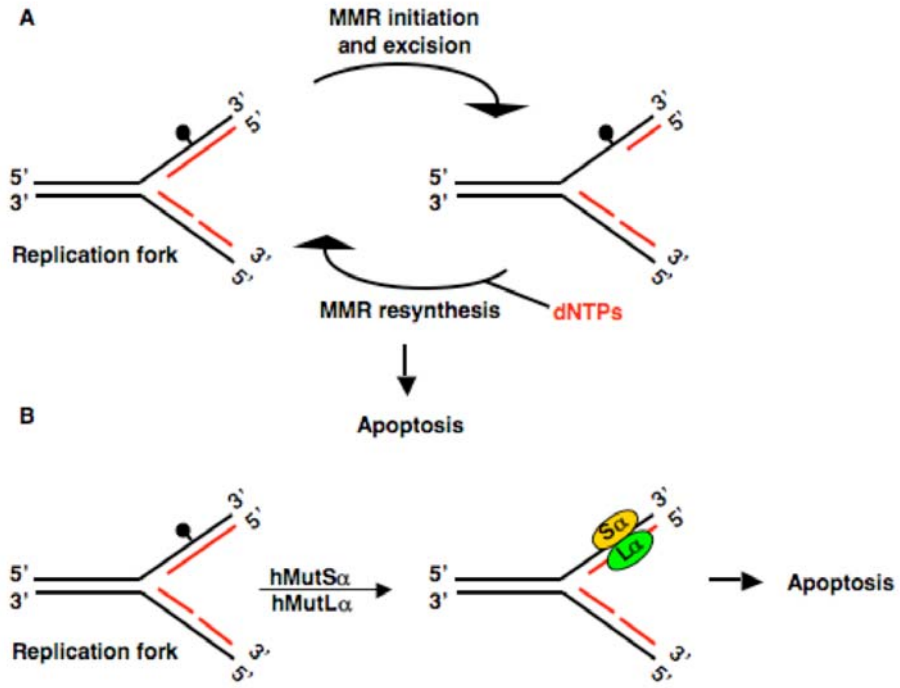

Figure 15 Models for MMR-dependent apoptosis in response to DNA damage.
A The "futile cycle" model
B The "sliding-clamp signaling" model (adapted from [321]).

A recent study demonstrates that up-regulation of MGMT activity and impaired MMR account at least in part for TMZ resistance in melanoma cells [322]. This is a little step forward to a better understanding of the mechanisms underlying primary and acquired drug resistance.

3.2.2 6-thioguanine (6-TG)

6-TG is an important drug used for the treatment of acute leukemia [323] and acts as an immunosuppressant in organ transplant patients [127]. After incorporation into DNA as 2'-deoxy-6-thioguanosine triphosphate [324], 6-TG residues are methylated *in vivo* by SAM to form S^6-methylthioguanine. During DNA replication, thymine or

cytosine can be incorporated opposite this modified base with roughly equal probability, resulting in 6-TG/T mismatches which are recognized by MutSα [325]. The substantial similarity of the miscoding properties and the cytotoxic action between O^6-methylguanine and 6-TG was confirmed by the findings that MMR-deficient, O^6-methylguanine-resistant cells generally exhibit ~10-fold cross-tolerance to the antimetabolite 6-TG [326-329].

3.2.3 Cisplatin

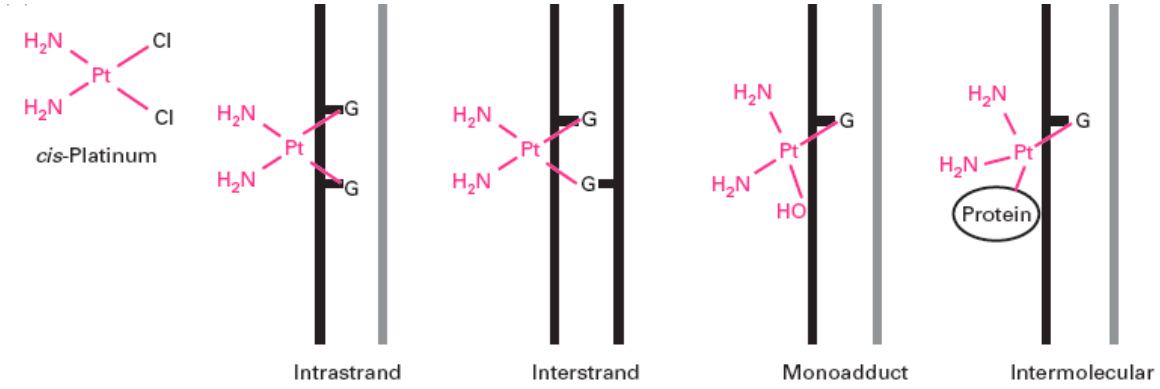

Figure 16 In the cells, cisplatin is converted into a charged electrophilic drug that reacts with DNA to form intrastrand and interstrand crosslinks, monoadducts and DNA-protein crosslinks (from [330]).

Cisplatin or CDDP (*cis*-diamminedichloroplatinum(II)) is an important drug used in the treatment of many tumors ([331]), especially of testicular and ovarian cancers [332]. It forms DNA-protein cross-links, DNA monoadducts, and both interstrand and intrastrand crosslinks (**Fig. 16**). 1,2-d(GpG) and 1,2-d(ApG) intrastrand crosslinks formed between neighbouring purine bases and representing 90% of the adducts are thought to be the key lesions in cisplatin-mediated cytotoxicity [333-335]. Although, in human cells these bulky structures are mainly addressed by nucleotide excision repair (NER), there is some evidence that MMR might contribute to the repair of cisplatin-induced DNA damage as well.

First, the 1,2-d(GpG) intrastrand crosslink in oligonucleotides is recognized and bound by MutSα [107]. Second, restoration of MMR capacity in human and mouse cells results in a concomitant two- to three-fold sensitivity to cisplatin [336-339].

One possible scenario could be that MMR factors recognize cisplatin adducts after DNA translesion polymerases bypassed the lesion and incorporated an incorrect

nucleotide [340-343]. NER would be competitively impeded to repair the damage by the bound MMR complex. This could trigger futile repair processes in a way similar to that described for O^6-methylguanine mispairs, eventually resulting in cell death (reviewed in [330]).

3.2.4 Topoisomerase inhibitors

Topoisomerase I (TOP1) enzymes are required to relax DNA supercoiling generated by transcription, replication and chromatin remodeling [344]. Anticancer drugs can trap TOP1 during their cleavage reaction, thereby reversibly stabilizing the so-called "cleavage complex" [345]. The cytotoxic activity of TOP1 inhibitors is related to the interference of the trapped complexes with DNA replication and transcription (reviewed in [346], **Fig. 17**).

3.2.4.1 Camptothecin and its derivatives

Camptothecin is a natural alkaloid extracted from the bark of the Chinese tree *Camptotheca acuminata* [347], with TOP1 as the only cellular target [348, 349]. Camptothecin-derivatives are in clinical use: Irinotecan is approved for colon carcinomas (usually in combination with 5-fluorouracil and leucovorin), whereas topotecan is indicated for the treatment of ovarian cancers. Although both drugs target TOP1 in a similar way, empirical values from clinical trials recommend different indications.

One group reported that MMR-deficient human colorectal cancer cells were much more sensitive to camptothecin than their proficient counterpart [350]. This study was carried out in a non-isogenic system, and indeed, results from our lab obtained with the strictly isogenic 293TLα cell line [351] did not show any difference between MMR-deficient and –proficient background [339].

However, severe side-effects and other limitations of camptothecins in cancer therapy promote the development of new derivatives and non-camptothecin TOP1 inhibitors [352, 353].

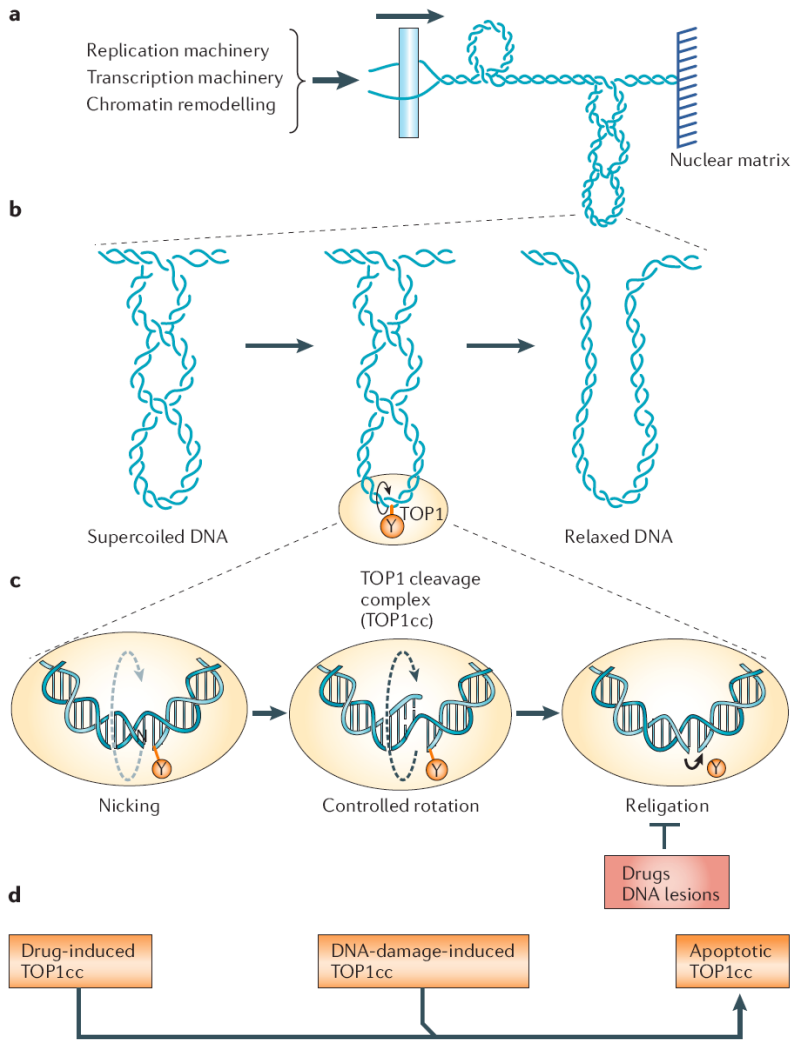

Figure 17 Relaxation of DNA supercoiling by TOP1-mediated DNA cleavage complexes, and the trapping of TOP1 cleavage complexes by drugs, DNA modifications and during apoptosis.
a The generation of DNA supercoiling by DNA replication, transcription and chromatin remodelling. The unwinding of duplex DNA by macromolecular complexes tracking along the DNA (arrow) without rotating freely around the DNA double helix, which is also unable to rotate freely owing to its length or attachment to nuclear matrix regions, generates positive supercoiling ahead of the unwound segment and negative supercoiling behind (negative supercoiling not shown).
b The introduction of DNA single-strand breaks (nicks) by TOP1 provides swivel points that enable the rotation of the intact DNA strand around the break and facilitate DNA relaxation. The cleavage intermediate is referred to as a cleavage complex because TOP1 cleaves DNA by forming a covalent bond to the 3′ DNA terminus that it generates. The covalently linked catalytic tyrosine of TOP1 (Y723 for human TOP1) is shown as the yellow circle.
c An expanded view of DNA relaxation by a TOP1 cleavage complex (TOP1cc). The first step (left) is a transesterification reaction whereby the catalytic tyrosine (Y) becomes linked to the 3′ DNA end (nicking step). In the second step (middle), the torsional strain that results from DNA supercoiling drives the rotation of the 5′ end of the nicked DNA strand around the intact strand. TOP1 encircles the rotating nicked DNA and slows its rotation. This process is referred to as ‘controlled rotation’. In the last step (right), the 5′ end of the nicked DNA is realigned with the corresponding 3′ end, which enables DNA religation (the closing step of the ‘nicking–closing reaction’). TOP1ccs are normally transient because the closing step is much faster than the nicking step. Drugs and DNA lesions inhibit religation by misaligning the ends of the broken DNA.
d TOP1ccs can be stabilized under three conditions: by drugs such as camptothecin (left), by DNA lesions (damage) that misalign the 5′ end of the nicked DNA, and by DNA and TOP1 modifications that occur during programmed cell death (apoptosis) (from [346]).

3.2.5 5-fluorouracil (5-FU)

The 5-fluoropyrimidine 5-fluorouracil (5-FU) is an antimetabolite drug that is widely used for treatment of cancer, particularly for colorectal cancer, with response rates of 10-20% only.

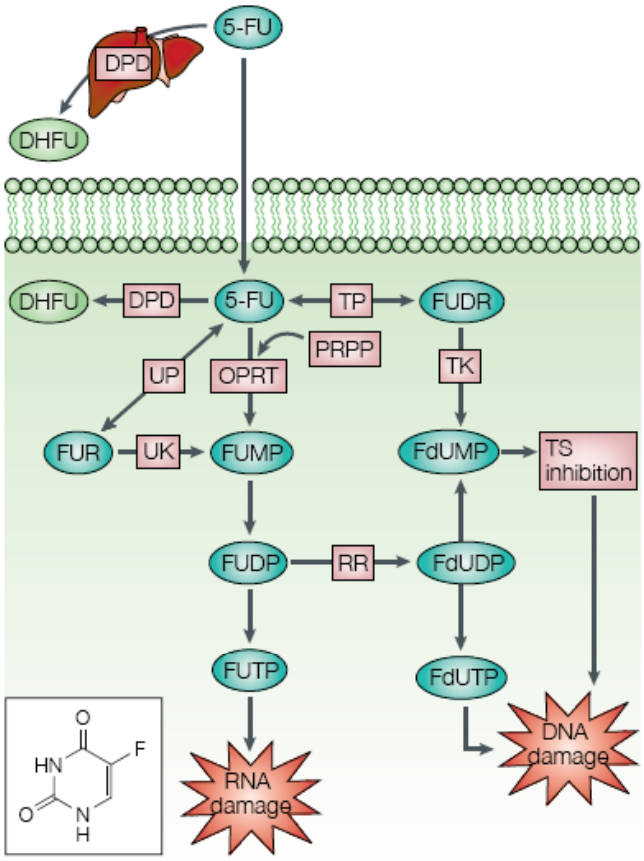

Figure 18 5-fluorouracil metabolism. 5-fluorouracil (5-FU; see structure) is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver (from [354]).

To increase the effectiveness of the therapy, 5-FU is usually combined and administered with other chemotherapeutic agents, such as irinotecan and oxaliplatin (a platinum-based cross-linker with a different spectrum of action and toxicity than cisplatin [355]) [356].

5-FU exerts its action in the form of three different metabolites: FdUrd-5'-monophosphate (FdUMP) inhibits DNA synthesis by blocking the activity of

thymidylate synthase (TS), 5-fluorouridine-5'-triphosphate (FUTP) is a precursor for RNA incorporation, and FdUrd-5'-triphosphate (FdUTP) is incorporated into DNA as FdUMP (**Fig. 18**).

Human MutSα was shown to recognize oligonucleotides containing 5-FU/G in both ATPase activity assays [357] and bandshift experiments (Fischer *et al.*, manuscript submitted). In contrary to these results, another group published the recognition of 5-FU/A by the MMR factor hMutSα [358]. But the inability of hMutSα to recognize 5-FU/A *in vitro* may not reflect its ability to recognize the lesion *in vivo* (see ***Results***, Fischer *et al.*, manuscript submitted). In addition, it has been reported that MMR-deficient cells are resistant to the cytotoxic effects of fluoropyrimidines [357, 359], which again emphasizes the important role of the MMR machinery in response to DNA-damaging agents.

3.2.5.1 TS inhibition and incorporation of fluoropyrimidines into DNA

TS directs the *de novo* synthesis of thymidylate (dTMP) from deoxyuridylate (dUMP) by transferring a methyl group from its cofactor (5,10-methylene tetrahydrofolate (THF)) to the C5 position of dUMP. In the presence of FdUMP, the ternary complex consisting of DNA, TS and THF is greatly stabilized due to the strong carbon-fluorine bond in the FdUMP molecule [360]. Consequently, depletion of dTMP induces significant perturbations in the levels of the other deoxynucleotides [361, 362], the concentrations of which are critical for determining the fidelity of DNA replication [363].

Meyers and colleagues provided evidence that the fluoropyrimidine-induced, MMR-mediated cytotoxicity is DNA-directed [357], and completed this finding by showing that DNA incorporation rather than TS inhibition is the responsible cytotoxic mechanism [364]. This is in agreement with results from a group working on the glycosylases UNG and SMUG1 and their roles in 5-FU processing [95]. Their most recent publication underscores the contribution of 5-FU incorporation into DNA to 5-FU-mediated toxicity, but ascribe the major role to SMUG1.

3.2.5.2 Incorporation of fluoropyrimidines into RNA

According to a widespread, longlasting notion, the key mechanisms causing cytotoxic effects are predominantly RNA incorporation and, to a minor extent, TS inhibition, but this idea may have to be reconsidered. The conclusion grew on a broad experimental base, with a number of publications providing evidence for the toxicity due to exclusive RNA incorporation. Furthermore, 5-FU incorporation into DNA is only 10% of the amount incorporated into RNA (reviewed in [365]).

Taking the complex and multilayered mode of action of fluoropyrimidines into account, the most probable scenario may propose pleiotropic effects of the drugs within the cell.

3.2.5.3 Clinical applications

Due to the above described resistance of MMR-deficient cells to 5-FU and its derivatives, one would expect that fluoropyrimidine treatment of these cells may is detrimental. Indeed, three clinical reports could confirm that 5-FU-based chemotherapy given to colon cancer patients with high levels of MSI did not result in a significant survival advantage [366-368].

In general, 5-FU/leucovorin, irinotecan and oxaliplatin are available for treatment of advanced colorectal cancer. The coupling of 5-FU with leucovorin, a reduced folate that increases thymidylate synthase inhibition, improves clinical outcome; these two drugs are therefore generally used in combination [369]. Recent clinical trials recommend the so-called FOLFOX regimen, a combination of oxaliplatin and infused fluorouracil plus leucovorin as a standard therapy for patients with advanced colorectal cancer [370].

The latest trials combine 5-FU with bevacizumab, an angiogenesis inhibitor, hoping for a new avenue in combination therapy. This drug targets VEGF (vascular endothelial growth factor), a key mediator of angiogenesis, thus choking off the blood supply that is essential for the growth of the tumor and its metastasis [371].

But as long as we do not know the exact mechanism(s) of MMR-dependent death after fluoropyrimidine exposure we are left with hope and a lot of scientific work…

Other agents whose cellular response has been shown to be affected by MMR and which are not discussed within the scope of this work, include oxidizing agents and IR [372, 373] that form 8-oxoguanine adducts in DNA [374, 375] and two additional topoisomerase inhibitors, doxorubicin and etoposide [350]. However, in our isogenic system, no differences in sensitivity of the 293TLα+ and 293TLα- cells to mitomycin C, chloroethylcyclohexyl nitrosourea (CCNU), melphalan, psoralen-UVA (interstrand cross-link agents), camptothecin, etoposide, or IR could be observed [339].

4. Aim of my studies

The present thesis can be divided into two major projects with different goals.

On the one hand, we intended to solve the riddle about the scenario “When MMR meets O^6-methylguanine” by using an *in vitro* MMR system with heteroduplex substrates containing one single O^6-methylguanine. Unfortunately, we encountered many problems and pitfalls during our work and we could not prove the MMR-dependent futile cycle hypothesis.

On the other hand, we exploited our *in vitro* system to find an answer to the contribution of the MMR system to the repair of 5-FU-containing mismatches. Since this lesion is addressed by both MMR and BER, we studied the participation of the individual candidates. We could nicely demonstrate that TDG is the key player in the repair of 5-FU/G mispairs, assisted by UNG. MBD4 did not contribute significantly to the repair events and SMUG1 turned out to be inactive under the experimental conditions. In the absence of glycosylases, MMR recognized and processed 5-FU/G to the same extent as G/T mismatches, implying an active competition between the two repair pathways. These findings, although demanding for further *in vivo* studies, may point to new approaches for the treatment of colorectal cancer by regarding the presence or absence of the appropriate glycosylases.

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6. Results

The results are presented as manuscripts or publications

6.1 5-fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems

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5-Fluorouracil is Efficiently Removed from DNA by the Base Excision and Mismatch Repair Systems.

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Abstract

Background & Aims: **5-Fluorouracil (FU) is one of the mainstays of colon cancer chemotherapy. Although developed as an inhibitor of thymidylate synthase (TS), its mode of action is complex. Thus, in addition to killing cells through thymidine depletion, FU cytotoxicity has been linked also to incorporation into RNA. Surprisingly, little is known about the consequences of FU incorporation into DNA.**
Methods: **Using extracts of human cells and circular DNA substrates containing a single FU residue either paired with adenine or mispaired with guanine, we studied the efficiency and directionality of FU removal.**
Results: **In nicked circular substrates, FU/G mispairs were efficiently repaired by MMR. In covalently-closed circular DNA, FU/G repair was initiated by either thymine DNA glycosylase (TDG) or uracil DNA glycosylase (UNG), whereas FU/A pairs were processed by UNG. Methyl-CpG binding domain protein 4 (MBD4) and single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1) did not detectably contribute to FU removal, however, as these recombinant enzymes process, FU/G and FU/A in oligonucleotide substrates, respectively, they too may be involved in FU metabolism *in vivo*.**
Conclusions: **The functional redundancy of MMR and DNA glycosylases in FU processing should ensure that the drug is efficiently removed from DNA before it can interfere with essential metabolic processes. However, in FU-treated cells, the nucleotide pools are depleted of thymine. The repair synthesis might thus be inhibited and leave cytotoxic gaps or breaks in DNA. Moreover, FU and/or FdUMP removed from DNA will increase the intracellular concentration of the drug, and thus exacerbate its cytotoxicity.**

Abbreviations: BER, base excision repair; BSA, bovine serum albumin; CRC, colorectal cancer; FU, 5-fluorouracil; FdUMP, 5-fluorouracil-2'-deoxy-5'-monophosphate; FdUTP, 5-fluorouracil-2'-deoxy-5'-triphosphate; MMR, mismatch repair; SMUG1, single-strand selective monofunctional uracil-DNA glycosylase 1; TDG, thymine DNA glycosylase; TS, thymidylate synthase; UNG, uracil DNA glycosylase;

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Introduction

Colorectal cancer (CRC) is the third most frequent malignancy in the Western world and accounts for 10% of all new cancer cases and cancer-related deaths in the United States (for review see [1]).

Developed in the late fifties as inhibitors of thymidylate synthase (TS), a key enzyme of *de novo* pyrimidine biosynthesis[2, 3], the fluoropyrimidine 5-fluorouracil (FU) and its nucleoside derivative 5-fluoro-2′-deoxyuridine represent the mainstay of chemotherapy of CRC, as well as of a variety of other types of cancer. However, although FU is an important chemotherapeutic, its mode of action is more complex than originally believed. The imbalance of dNTP pools caused by TS inhibition was shown to slow down DNA replication[4], and because the efficacy of FU therapy is potentiated by leucovorin, which stabilizes the FU/TS complex with methylenetetrahydrofolate[5], this mode of action is believed to be clinically relevant. However, FU was reported to exert its cytostatic effect also through incorporation into RNA, where it was suggested to interfere with RNA processing[6, 7] and it has been proposed that incorporation of 5-fluoro-2′-deoxyuridine-5'-monophosphate (FdUMP) into DNA is also cytotoxic[8].

When attempting to elucidate the mode of action of a cancer chemotherapeutic, invaluable lessons can be learned from the study of drug resistance, both inherent and acquired. Tumors resistant to FU are commonplace, as are FU-resistant tumor-derived cell lines (see ref. [1] for review). Analysis of these cells showed that FU resistance is often linked to an upregulation of expression of TS, which means that higher drug concentrations are required to inhibit the larger cellular TS pool, or to elevated levels of dihydropyrimidine dehydrogenase (DPD), which catabolises the drug. An alternative path to resistance is to express low levels of thymidine kinase (TK), which will result in low FdUMP levels and thus in low TS inhibition. However, FU resistance was correlated also with low uridine-cytidine monophosphate kinase (UMPK) levels. This enzyme converts FdUMP and FUMP into the corresponding diphosphates, which are then further phosphorylated to the triphosphates FdUTP and FUTP, substrates of DNA and RNA polymerases, respectively. The link between low UMPK levels and FU resistance confirmed the hypothesis that incorporation into nucleic acids is harmful. However, recent reports describing the lack of response to FU therapy in patients with tumors displaying microsatellite instability (MSI), a hallmark of mismatch repair (MMR) deficiency, implied that removal of FU from DNA by the MMR system is more toxic than when the base remains unrepaired.

MMR improves the fidelity of DNA replication by several orders of magnitude, through correcting biosynthetic errors in newly synthesized DNA. It also controls DNA recombination by helping to abort strand exchange between non-identical sequences. More recently, MMR has been implicated also in the processing of modified nucleotides and DNA damage signaling and apoptosis, as MMR-deficient cells are more resistant than matched MMR-proficient ones to several different classes of chemicals (recently reviewed in [9, 10]). As noted above, the MMR system was suggested to play a role also in the cellular response to FU treatment[11-14]. In three of these publications, patients with MSI^+ CRCs were reported not to benefit from FU-based chemotherapy, and these findings appeared to be confirmed by *in vitro* studies[8, 15, 16], which showed MMR-deficient cells to be more resistant to FU than matched MMR-proficient ones. What these studies implied is that removal of FU from DNA by the MMR system is deleterious to the cells, which runs contrary to the above hypothesis. However, the reason underlying the apparent lack of response of MMR-deficient tumors and cells to FU was not investigated in detail in these publications. One study ascribed it to a higher expression of TS in MMR-deficient cancers[17], but this was not confirmed in another laboratory[18]. Moreover, clinical data from Finland[19] suggest that MSI^+ tumors respond better to FU treatment than MSI^- CRCs. In an attempt to learn more about the role of MMR in the cytotoxicity of FU, we set out to study the processing of this base analogue in DNA.

Unlike the other halogenated pyrimidines 5-bromouracil and 5-iodouracil, FU does not readily undergo a tautomeric shift to the enol form (Fig. 1A) and is therefore unlikely to be misincorporated into DNA opposite guanosine during replication with appreciable frequency. We anticipated that, in the event of this happening, the resulting FU/G mispair would be corrected by the MMR system to a C/G. However, given that FU will predominantly form FU/A pairs, which very closely resemble T/A pairs, we wanted to test whether these latter structures were also recognized and processed by the MMR system. Moreover, given that the fluorine atom at the 5-position of the pyrimidine has a van der Waals radius similar to a proton, FU is a substrate for all four known human DNA glycosylases that remove uracil from DNA: uracil-DNA glycosylase (UNG)[20], thymine-DNA glycosylase (TDG)[21, 22], single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1)[23] and methyl-CpG binding domain protein 4 (MBD4)[24, 25] (reviewed in [26]). However, the relative efficiency of FU processing by these glycosylases has not been tested in a system where all four were present at the same time and where also the MMR system was functional.

In this study, we show that FU/G and, to a lesser extent, FU/A base pairs incorporated into circular DNA substrates are addressed by the MMR system in extracts of human cells. In

addition, the former substrate is very efficiently repaired to C/G by base excision repair (BER). In this processing, TDG plays the predominant role. The processing of FU/A by BER could be shown to involve UNG, but SMUG1 and TDG may also play important roles in the processing of this most frequent lesion.

Materials and Methods

Substrates, nuclear extracts and in vitro mismatch repair (MMR) assays

The detailed procedure has been described previously[27]. Briefly, heteroduplex DNA substrates containing a FU/A or a FU/G mismatch within an *Acl*I restriction site in the 46 bp polylinker of a pGEM13Zf(+) derivative were constructed by primer extension, using an FU-containing oligonucleotide as the primer and the single-stranded phagemid DNA as template. The desired supercoiled heteroduplex substrates were purified by caesium chloride centrifugation. The strand discrimination signal was introduced by incubation with *N.Bst*NBI, which introduced a specific nick in the complementary strand (3' from the mismatch) at position 352 of the duplex. The mismatch repair assays were performed in 20 m*M* Tris-HCl pH 7.6, 110 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* glutathione, 50 μg/ml BSA, 0.1 m*M* each dGTP, dCTP, dTTP and dATP, 1.5 m*M* ATP, 100 ng (47.5 fmol) heteroduplex DNA substrate and 50 μg of nuclear extract from 293T-Lα+ ($MLH1^+$), or 293T-Lα- ($MLH1^-$)[28] cells in a total volume of 25 μl. After 30 min incubation at 37°C, the reactions were terminated by the addition of a stop solution and further incubation for 30 min at 37°C (final concentrations: 25 m*M* EDTA, 0.67% SDS, 50 μg/ml proteinase K). The DNA was purified using Qiagen MinElute Reaction Cleanup Kit, digested with the appropriate mismatch-discriminating restriction enzyme and treated with 50 μg/ml RNase A, followed by proteinase K (60 μg/ml) in the presence of 0.2% SDS. The DNA was then precipitated in 0.3 *M* NaOAc pH 5.5/70% ethanol, and resuspended in 10 m*M* Tris-HCl. The efficiency of the repair reactions was monitored on 1% TAE agarose (Invitrogen) gels stained with ethidium bromide.

Some assays contained also 1.7 pmol [α-^{32}P]dATP (8.5 μCi). To monitor incorporation of the radiolabeled nucleotide, the agarose gels were vacuum-dried and exposed to a PhosphoScreen (Molecular Dynamics, Inc.).

Immunodepletion of nuclear extracts

Dynabeads®Protein A (Dynal Biotech, Invitrogen) were washed twice with 30 m*M* Hepes-KOH pH 7.5, 7 m*M* $MgCl_2$ and incubated for 2 h at 4°C with either anti-MBD4 (Sigma,

1:2000) or anti-TDG (rabbit polyclonal, a kind gift of Primo Schär, 1:10000) antibodies. After washing three times with the above buffer, the pre-adsorbed beads were stored at 4°C until required. 50 μg of nuclear extracts were incubated for 30 min at 4°C with 6.25 μl of the antibody-pre-adsorbed Dynabeads and subsequently used for *in vitro* MMR assays or Western Blots.

Bandshift assays

40 fmol (2 nM) of annealed 5'- [^{32}P]-labeled oligonucleotides (TTT CTG ACT TGG ATA CCA **FU**CT ATC TAT CTA TAA AAT AT, TTT CTG ACT TGG ATA CCA **T**CT ATC TAT CTA TAA AAT AT, ATA TTT TAT AGA TAG ATA G**A**T GGT ATC CAA GTC AGA AA, or ATA TTT TAT AGA TAG ATA G**G**T GGT ATC CAA GTC AGA AA (all written 5' to 3' – see ref. [29]) were incubated with 100 m*M* KCl, 25 m*M* Hepes-KOH pH 7.5, 1 m*M* DTT, 0.5 m*M* $MgCl_2$, 0.1 m*M* ADP, 10% glycerol, 75 μg/ml BSA and 60 ng poly(dI-dC)•poly(dI-dC) competitor (Amersham Pharmacia Biotech) in the presence of 40, 70, or 100 n*M* purified human MutSα in a final volume of 20 μl. After 20 min at 37°C, 10 μl were loaded onto 9x8 cm 5% native polyacrylamide (29:1) gels (ATTO system) eluted with 1x TAE buffer and run 30 min at 19 V/cm. The vacuum-dried gels were exposed to PhosphoScreens (Molecular Dynamics, Inc).

Nicking assay

50 fmol (2.5 nM) of annealed 5'-[^{32}P]-labeled oligonucleotides (TTT CTG ACT TGG ATA CCA **FU**CT ATC TAT CTA TAA AAT AT, ATA TTT TAT AGA TAG ATA G**A**T GGT ATC CAA GTC AGA AA, ATA TTT TAT AGA TAG ATA G**G**T GGT ATC CAA GTC AGA AA, all written 5' to 3' – see ref. [29]) were incubated either under MMR conditions [20 m*M* Tris-HCl pH 7.6, 110 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* glutathione, 50 μg/ml BSA, 0.1 m*M* each dGTP, dCTP, dTTP and dATP, and 50 μg of nuclear extract from 293T Lα^- (MLH1$^-$)] or under standard conditions (20 m*M* Tris-HCl pH 8, 1 m*M* EDTA, 1 m*M* DTT, 0.1 mg/ml BSA) in the absence or presence of UGI (1U) and/or SMUG1 (4U) in a final volume of 20 μl. Reactions were incubated for 1 h at 37°C followed by a denaturing step for 5 min at 95°C in the presence of 0.1 *M* NaOH. After adding 20 μl 8 *M* urea/1xTBE loading buffer, 10 μl were loaded onto a pre-run 15% denaturing PAGE (8 *M* urea) and ran at approximately 45 V/cm for 20 min. Finally, the gels were exposed to a PhosphoScreen (Molecular Dynamics, Inc.).

Results

Binding of MutSα to FU/G and FU/A

To test the ability of the MMR machinery to recognize FU-containing base pairs in DNA, we incubated 38-mer oligonucleotides containing a T/A, T/G, FU/A or FU/G base pair at position 19[29] with varying concentrations of the purified recombinant human mismatch binding factor MSH2/MSH6 (MutSα). As anticipated, these experiments revealed that MutSα binds the FU/G substrate with an affinity similar to T/G. In contrast, FU/A and T/A substrates failed to form stable protein/DNA complexes in this assay, even at high concentrations of MutSα (Fig. 1B). Our findings agree with the data of Meyers *et al.*[16, 26], but are in sharp contrast to those of Tajima and colleagues[29], even though both sets of experiments were carried out with identical oligonucleotide sequences.

Processing of FU-containing base pairs in nuclear extracts of human cells

Our previous experience showed that mismatches that are efficiently bound by MutSα in bandshift experiments are efficiently repaired by the MMR system in *in vitro* MMR assays. However, the contrary is not necessarily true. We therefore wanted to test the efficiency with which the FU/A and FU/G pairs are addressed in a mismatch repair assay. To this end, we constructed circular heteroduplex substrates, in which the FU/A or FU/G base pairs were embedded within the recognition site of the *Acl*I endonuclease (Fig. 2A). In order to be able to follow the progress of the *in vitro* repair process, the presence of the modified base or mismatch has to inhibit the cleavage of this site by the restriction enzyme. Successful MMR outcome is then scored as the amount of DNA converted to an *Acl*I-susceptible form.

As shown in Fig. 2B, the presence of a T/G or FU/G mispair at this site rendered the substrates refractory to cleavage with the enzyme. Thus, these substrates can be used in the *in vitro* MMR assays with *Acl*I. In contrast, the FU/A substrate was as efficiently cleaved by the enzyme as the control T/A plasmid. Taken together with the result of the bandshift assay shown above, this result confirmed that the FU/A base pair structurally resembles a T/A. As this result precluded the use of *Acl*I digests as readout of FU/A repair efficiency, the repair assays with the latter substrates had to be followed by incorporation of radiolabeled nucleotides.

Successful mismatch correction in human cell extracts requires that the circular heteroduplex substrates carry also a strand discontinuity within ~1 kb upstream or downstream from the mispair. This is the site where the exonucleolytic mismatch correction

process initiates. Our plasmids contain a single *N.Bst*NBI site 297 base pairs from the mismatch. This enzyme recognizes the sequence GAGTC/CTCAG, but cleaves the DNA only in the top strand. Covalently-closed circular heteroduplex substrates, which are refractory to mismatch correction *in vitro*, were used as controls where appropriate.

When the G/T substrate was incubated with mismatch repair-deficient extracts of 293T-Lα cells, no repair was observed. In contrast, in the presence of mismatch-repair proficient extracts, approximately 60% of the heteroduplex substrate were converted into an *Acl*I-cleavable form that is indicative of G/T to A/T repair, as shown by the appearance of the 1.5 and 1.3 kb bands in the agarose gel (Fig. 2C, lanes 5 and 6, respectively). As anticipated, the FU/A plasmid was completely cleaved after incubation with the extracts (lanes 1 and 2), and this result was therefore uninformative (but see Fig. 5). The efficiency of FU/G to C/G repair was similar to G/T in the MMR-proficient extract (compare lanes 4 and 6). Surprisingly, however, we observed an appreciable extent of FU/G repair also in the MMR-deficient 293T-Lα extract (lane 3). As the FU/G mispair was addressed to a similar extent also in a covalently-closed substrate that is refractory to MMR (data not shown), we assumed that the FU residue was recognized and repaired by BER.

Contribution of UNG and MBD4 to FU/G repair in vitro

FU was reported to be processed by UNG[30] and MBD4[31]. As the latter studies were carried out with synthetic oligonucleotide substrates and purified glycosylases in isolation, we set out to test, which of these enzymes was responsible for processing of the FU/G mismatch in a circular plasmid substrate in the 293T-Lα extracts.

We incubated the MMR-deficient nuclear extract of 293T-Lα$^-$ cells with increasing concentrations of the uracil glycosylase inhibitor (UGI) (Fig. 3A). Although the tested amounts of UGI were sufficient to inhibit the UNG activity in these nuclear extracts when tested on radiolabeled oligonucleotides in nicking assays (data not shown), the inhibition of FU/G processing in our circular substrate was barely detectable. We therefore concluded that UNG was either not required for FU processing, or that another activity in the extracts, possibly MBD4, was compensating for the lack of active UNG.

When we immuno-depleted the 293T-Lα$^-$ nuclear extract of MBD4 by a procedure (see Materials and Methods) that reduced the amount of MBD4 in the extracts to an almost undetectable level (Fig. 3B, left panel, lanes 1 and 2), the efficiency of MMR did not diminish, as witnessed by the unchanged efficiency of G/T repair (right panel, lane 6). In

these extracts, the FU/G mispair processing (right panel, lane 3) was similarly efficient to that seen in undepleted extracts (right panel, lane 1), which indicated that MBD4 was either not responsible for FU repair under our experimental conditions, or that it was not active in the cell extracts.

TDG depletion reduces the efficiency of FU/G repair to C/G

TDG is known to remove U or T from U/G or T/G mispairs[32] and has been reported to efficiently remove FU residues from oligonucleotide substrates[33]. We set out to test whether this latter enzyme might be responsible for the observed FU/G processing in the circular substrates in the 293T-Lα extracts, by immuno-depleting them of TDG, using a rabbit polyclonal anti-TDG serum (a kind gift of Primo Schär). TDG exists in two different forms: the unmodified protein, which migrates in SDS-PAGE gels with an apparent molecular size of 55-60 kDa and the SUMOylated variant with an apparent molecular size of ~80 kDa[34]. The efficiency of FU/G repair in cell extracts depleted of both these TDG forms (Fig. 3C, left panel, lanes 1 and 2) was somewhat reduced (Fig. 3C, right panel, lane 3), indicating that TDG is at least partially involved in the repair of FU residues mispaired with G.

Lack of TDG, UNG and MMR abolishes repair of FU/G in vitro

Having observed that TDG and, to a lesser extent, UNG, contribute to the repair of FU/G mispairs, we postulated that these enzymes might be functionally redundant in FU processing. This indeed appears to be the case. In MMR-deficient extracts depleted of TDG (Fig. 4, left panel, lane 1), in which UNG was inhibited by the addition of UGI, a nearly complete abrogation of FU/G repair was observed (right panel, lane 3). Importantly, in the absence of TDG and UNG, the MMR machinery processes the FU/G mismatch with efficiency similar to that of the G/T control (right panel, lanes 4 and 6).

In summary, in our *in vitro* system, human TDG, UNG and MMR can all process FU/G mispairs. The base excision repair process initiated by TDG and UNG always converts the FU/G to a C/G. Unlike BER, MMR-mediated repair is directed to the strand carrying the nick, which is required for initiation of the excision. In the substrates shown in Figs. 2-4, which contained a nick 297 nucleotides 3' from the FU/G mispair in the FU strand, MMR catalyzed the conversion of the FU/G mismatch to C/G, similarly to BER. This reflects the situation when the FU is incorporated opposite G during replication. However, should a G be incorporated opposite an FU residue in the template strand, then the G/FU mispair would be repaired by MMR to an A/FU.

FU/A repair in human cell extracts

As shown in Fig. 2B (lane 1), the restriction enzyme *Acl*I is insensitive to the presence of FU in its recognition site. Given that the FU/A heteroduplex is completely cleaved by the enzyme, it cannot be used as a substrate to study the processing of FU/A by the above-described assays. However, by carrying out the repair reactions in the presence of [α-^{32}P]dATP, we could monitor the extent of enzymatic processing by the amount of radioactivity incorporated into the three restriction fragments generated by the *Acl*I digests. In the control experiment, we incubated the G/T substrate with MMR-deficient and –proficient cell extracts and confirmed that MMR-dependent G/T to A/T processing took place solely in the latter extracts (Fig. 5B, lanes 5 and 6, respectively). The autoradiograph of the same agarose gel revealed that most of the radioactivity was incorporated into the 1.5 and 1.3 kb fragments generated by the *Acl*I digest upon successful MMR-catalysed G/T to A/T repair. The fact that both these fragments were labeled with similar efficiency confirms the results of previous studies, which showed that the MMR repair tracts span the distance between the strand discontinuity (a nick situated in the G strand 297 nucleotides 3' from the mismatch in our case) and ~150 nucleotides past the mismatch[35]. The radioactivity incorporated into the uncleaved 2.8 kb fragment most likely represents a small amount of strand displacement commencing at the nick. In a MMR-deficient extract, G/T to A/T processing was hardly detectable (Fig. 5B, lane 5) and radioactivity was incorporated predominantly into the uncleaved 2.8 kb fragment, presumably also through strand displacement.

As anticipated, the FU/A substrates were completely cleaved by *Acl*I into the 1.5, 1.3 and 0.3 kb fragments. Although no differences between the assays using MMR-deficient and –proficient extracts could be seen in ethidium bromide-stained gels (Fig. 5B), autoradiography of the gels revealed interesting differences. When the MMR-deficient extract was used (Fig. 5C, lane 1), the radioactivity was incorporated predominantly into the 1.5 and 1.3 kb fragments in an approximate ratio of 3:1. When the same extract was depleted of TDG (Fig. 5A, lane 1) and supplemented with UGI to inhibit also UNG, the incorporation of radioactivity into the 1.5 kb fragment increased (Fig. 5C, lane 3). This suggests that the radioactivity was incorporated through DNA synthesis starting at the nick and proceeding away from the FU/A pair. This strand displacement reaction was inhibited by the presence of the two glycosylases.

In MMR-proficient extracts, both the 1.5 and 1.3 kb fragments contained similar amounts of incorporated radioactivity, which shows that the FU/A mispair was processed by the MMR system, giving rise to long repair tracts that flank the mispair on either side. Although the amount of radioactivity in these fragments (Fig. 5C, lanes 2 and 4) was substantially lower than that seen in the G/T substrate (Fig. 5C, lane 6), and although the MMR system was somewhat inhibited by the presence of TDG and UNG (Fig. 5C, compare lanes 2 and 4), our results clearly show that the MMR system recognizes and processes the FU/A substrate, albeit with low efficiency. This finding was unexpected, given that the FU/A oligonucleotide duplex was not appreciably bound by MutSα in the bandshift assay (Fig. 1B) and that the FU/A base pair apparently closely resembles a T/A; indeed, we failed to find a restriction enzyme capable of distinguishing between FU/A- and T/A-containing oligonucleotide substrates (data not shown).

The role of SMUG1 in the repair of FU-containing substrates

Mammalian cells possess, in addition to UNG, TDG and MBD4, one additional DNA glycosylase capable of processing uracil residues in DNA: SMUG1. This enzyme was initially believed to be specific for uracil in single-stranded DNA, hence its name - single-strand selective monofunctional uracil DNA glycosylase. However, it is now known to efficiently process also A/U and G/U substrates, at least at low salt concentrations *in vitro*[36].

As shown above (Figs. 2-5), inhibition of TDG and UNG activities in the absence of MMR was sufficient to abolish processing of the FU/G substrate in our *in vitro* assays. These results therefore suggested that, like MBD4, SMUG1 did not contribute significantly towards the processing of FU in DNA in our system. However, the protein was shown to be present in the 293T-Lα extracts in relatively high amounts, as shown by western blotting (Fig. 6A), so some processing of the FU-containing substrates was anticipated if SMUG1 were able to act on them. Indeed, when the recombinant enzyme was incubated with FU-containing oligonucleotide substrates, it processed both the FU/G and the FU/A substrates rather efficiently (Fig. 6B, lanes 6 and 7, 9 and 10**)**. We therefore suspected that SMUG1 might be inactive under the conditions of our *in vitro* MMR assay. This was indeed the case, as the enzymatic activity of the recombinant enzyme observed in the buffer recommended by the supplier was lost when the experiment was carried out in our MMR buffer (Fig. 6B, lanes 1-4; see *Materials and Methods*). As we cannot change the composition of this buffer without losing MMR activity, we are currently unable to test the contribution of SMUG1 towards FU processing in human cell extracts. However, we postulate that its activity *in vivo* would

contribute, together with UNG, TDG and, possibly also MBD4, towards the efficient removal of FU from DNA (see below).

Discussion

In spite of the fact that FU has been in daily use in clinical practice as an antimicrobial and anticancer agent for 50 years now, it's mode of action is still poorly understood, principally due to the complexity of its metabolism. Its function as an inhibitor of thymidylate synthase (TS) is beyond doubt; it has been shown to irreversibly inhibit this enzyme by forming a covalent intermediate between its deoxyribonucleotide FdUMP and TS, trapping thus also methylenetetrahydrofolate in the complex. Moreover, its clinical efficacy in colon cancer therapy is somewhat increased by leucovorin, which stabilizes this ternary complex. FU can be also metabolized to the ribonucleotide and be incorporated into RNA in the form of FUMP, where it is believed to interfere with the function of tRNA and rRNA, as well as with mRNA metabolism. Although it has been known for several years that substantial amounts of FdUMP are incorporated into DNA, the contribution of this component to FU cytotoxicity has been little studied to date. We now show that this base analogue is efficiently removed from DNA, irrespective of whether it is base paired with adenine or guanine. In the latter case, the FU/G mispairs are addressed by the MMR system, by TDG, UNG and most likely also by SMUG1. FU/A pairs are poor substrates for MMR, but are addressed by UNG and most likely also by TDG and SMUG1. Indeed, while this manuscript was in the last stages of preparation, a report from the Lindahl laboratory[37] showed that FU treatment of mammalian cells results in substantial levels of incorporation of this nucleoside analogue into DNA and that knock-down of SMUG1 by siRNA leads to some two-fold sensitization to the drug. These results demonstrate not only that FU residues are addressed by the latter enzyme, but also that the removal of FU from DNA by SMUG1 has a protective effect – in contrast to UNG deficiency, which does not alter the sensitivity of the cells to the drug[38].

What are the implications of these findings for FU-therapy? The first conclusion that could be made on the basis of our experiments is that FU would not reside in DNA for long, given that it can be removed by at least four enzymatic pathways. As it would be incorporated into newly-synthesized DNA predominantly opposite A, most of the removal would be expected to be catalyzed by SMUG1 and possibly also by UNG and TDG. Given that these enzymes process identical substrates, their functional redundancy would ensure that loss of one of these enzymes, e.g. through mutation or transcriptional silencing, would not carry

serious phenotypic consequences. Indeed, as mentioned above, UNG-deficient murine $Ung^{-/-}$ cells are not hypersensitive to FU[38]. In the case of FU incorporation opposite G, the resulting FU/G mispair would be efficiently processed by the MMR system. In its absence, it would be removed by the glycosylases TDG, UNG and probably also by MBD4 and SMUG1. In all these cases, the removal would not give rise to mutations, as the FU/G mispairs would be converted to C/G. Similarly to the case of the FU/A base pairs, it could be argued that the redundancy of the repair pathways addressing FU/G would make it unlikely that inactivation of any one would result in an increased sensitivity to the drug. However, this does not appear to be the case, given that SMUG1 deficiency sensitizes cells to FU. This implies that the different glycosylases contribute differently towards the removal of the halogenated base from DNA, as seen also in our assays, where TDG was shown to be notably more active on FU/G than UNG (compare Fig. 3A and 3C). Moreover, it also conceivable that the removal of FU from different genomic regions or during an inappropriate stage of the cell cycle may affect the cytotoxicity of the drug. Thus, UNG is believed to be associated predominantly with replication foci, while SMUG1 appears to be found predominantly in nucleoli[36] and TDG is degraded prior to S-phase[34]. In the absence of SMUG1, ribosomal DNA might be left largely unrepaired, which would cause inefficient synthesis of rRNA and inhibition of translation. In the absence of TDG, FU-containing DNA might persist until the following replication cycle, where removal of FU residues from the single stranded template DNA might result in replication fork collapse and subsequent apoptosis.

There is one more point worth considering. Our experiments were carried out in an *in vitro* system, in which the FU-containing substrates were incubated with nuclear extracts of human cells supplemented with all four nucleoside triphosphates. In this scenario, the excision of FU residues mediated by MMR or BER could be followed by DNA synthesis, where the repair tracts were filled-in by polymerase-δ or –β, respectively. In a cell treated with FU, however, the nucleotide pools are depleted of dTTP, which has two important consequences: first, the replicating polymerases will incorporate large amounts of FdUMP into nascent DNA, and second, the repair polymerases will be unable to replace FdUMP residues removed by MMR or BER with TMP and will thus re-incorporate FdUMP into the repair tracts, triggering thus further rounds of futile repair. Moreover, FU excised by BER, or FdUMP excised by MMR, would be recycled to find their way back into a complex with TS, or into the DNA. In this way, the DNA repair processes would continuously replenish the FU levels in the cells and thus augment the toxicity of the drug.

In conclusion, our experiments demonstrate that FU residues in DNA are efficiently removed by several redundant enzymatic systems. This processing could contribute substantially towards the toxicity of the drug. It could be argued that because the MMR repair tracts are substantially longer that those resulting from base excision, the absence of the MMR system would reduce the amount of repair DNA synthesis and thus attenuate the toxicity of FU through limiting all repair events to BER. However, MMR would be acting predominantly on FU/G mispairs, which would be expected to arise only rarely and the toxicity associated with MMR repair tracts would therefore be minimal. The differential sensitivity of the MMR-proficient and –deficient cell lines described in the literature[8, 15, 16] is thus likely to be the result of other genetic differences between the cell lines. Indeed, in our hands, the MMR-deficient cell lines HCT116 and 293T-Lα^{-28} are as sensitive to FU as the matched MMR-proficient lines HCT116+chr3 and 293T-La^{+} (data not shown). The open question concerns the *in vivo* role of TDG, which would be expected to participate in the removal of FU from both, FU/G and FU/A pairs. If this enzyme were as active in cells as in our *in vitro* assays, it would be expected to exert a substantial protection against FU cytotoxicity. Given that *Tdg* knock out mice are embryonic lethal and that no isogenic TDG-proficient and -deficient cell pairs exist, these experiments will have to await generation of such stable cell lines by, for example, shRNA technology.

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Legends to Figures

Fig.1 Chemical structures of halogenated pyrimidines and the recognition of FU in DNA by the human mismatch binding factor MutSα. *A*, Keto-enol tautomerism of uracils substituted with fluorine (FU), bromine (BrU) and iodine (IU). The question mark indicates the uncertainty regarding the prevalence of the enol form of FU. *B*, Binding of MutSα to FU/G and FU/A oligonucleotide substrates. The 5'-[^{32}P]-labeled oligonucleotides (A/T, G/T, FU/A, FU/G) were incubated with 40 nM (lanes 1-4), 70 nM (lanes 5-8) or 100 nM (lanes 9-12) of purified human MutSα. The figure is an autoradiogram of a 5% native polyacrylamide gel.

Fig.2 Processing of FU-containing base pairs in nuclear extracts of human cells. *A*, Schematic representation of the construction of the FU-containing DNA substrates and of the *in vitro* MMR assay. *B*, Susceptibility of FU-containing heteroduplex substrates to cleavage with *Acl*I. The FU/A (lane 1) substrate was cleaved at position 46 (see panel *A* above), whereas the FU/G (lane 2) and G/T (lane 3) substrates were refractory to cleavage at this position. *C*, *In vitro* MMR assays performed with the FU/A (lanes 1 and 2), FU/G (lanes 3 and 4) or G/T (lanes 5 and 6) substrates. The 1516 bp and 1307 bp restriction fragments are indicative of repair (for details see 2A). The reactions were carried out in nuclear extracts of MLH1-proficient (+), or MLH1-deficient (-) 293T-Lα cells.

Fig.3 Contribution of different glycosylases to the repair of FU/G mispairs. *A*, mismatch repair assay in the presence of different concentrations of uracil DNA glycosylase inhibitor (UGI). The reactions were performed in nuclear extracts of MLH1-proficient (+), or MLH1-deficient (-) 293T-Lα cells, with the nicked FU/G (lanes 1-5) or G/T (lane 6) substrates. The used UGI concentrations [U] are indicated in units defined by the manufacturer. *B*, Effect of MBD4 depletion on FU/G repair. Efficiency of MBD4 immunodepletion from 293T-Lα nuclear extracts was confirmed by Western blot analysis (MBD4$^-$, left panel, lanes 1 and 2). The MMR assays (right panel) were performed with undepleted (lanes 1 and 2) or MBD4-

depleted nuclear extract (lanes 3-6). The DNA bands representing the repaired fragments were quantified using ImageQuant TL, Amersham Biosciences (right bottom panel). *C*, Effect of TDG depletion on FU/G repair. The reactions are identical to those shown in panel *B*, except that both SUMOylated and unmodified forms of TDG (arrows) were immunodepleted.

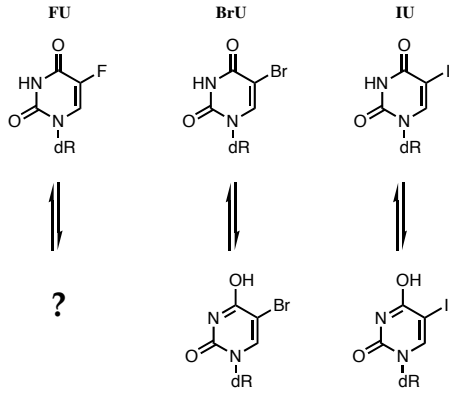
Fig.4 Lack of TDG, UNG and MMR abolishes FU/G repair *in vitro*.
The immunodepletion and Western blot analyses are identical to those shown in Fig. 3 *BC* (left panels). The MMR assay (right panel) was performed in the presence of UNG and TDG (lane 1), in the absence of UNG only (lane 2) or in the absence of both UNG and TDG (lanes 3-6).

Fig.5 FU/A repair in human cell extracts. *A*, Immunodepletion and Western blot analyses were identical to those shown in Fig. 3 *BC*. *B*, *In vitro* MMR assays supplemented with [α-^{32}P] dATP were performed in the presence (lanes 1 and 2) or absence of UNG and TDG (lanes 3-6). Radioactive incorporation was visualized with a PhosphorImager (*C*).

Fig.6 Processing of FU-containing substrates by SMUG1. *A*, SMUG1 is present in the extracts of 293T-Lα cells in large amounts, as shown by Western blot analysis. LoVo extracts, which express lower amounts of SMUG1 mRNA than 293T-Lα cells as judged by microarray experiments (J. Sabates-Bellver, unpublished) contain correspondingly lower amounts of the protein. *B*, *In vitro* activity of SMUG1 on FU-containing oligonucleotides. The FU/A and FU/G substrates were incubated either under MMR conditions (10xMMR buffer, lanes 1-4) or in buffer recommended by the manufacturer (10xDDR buffer, lanes 5-10, see *Materials and Methods*) in the presence (+) or absence (-) of MLH1-deficient nuclear extract of 293T-Lα cells (NE MLH1$^-$). As shown, the recombinant enzyme is inactive in the MMR buffer, which might explain the absence of processing of FU-containing substrates in the cell extracts. The figure is an autoradiograph of a 12% denaturing polyacrylamide gel.

Fischer *et al.*, Figure 1

A



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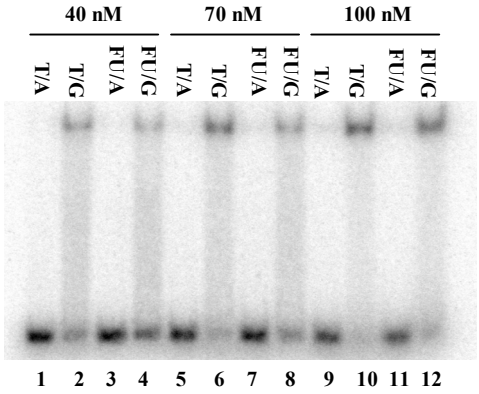
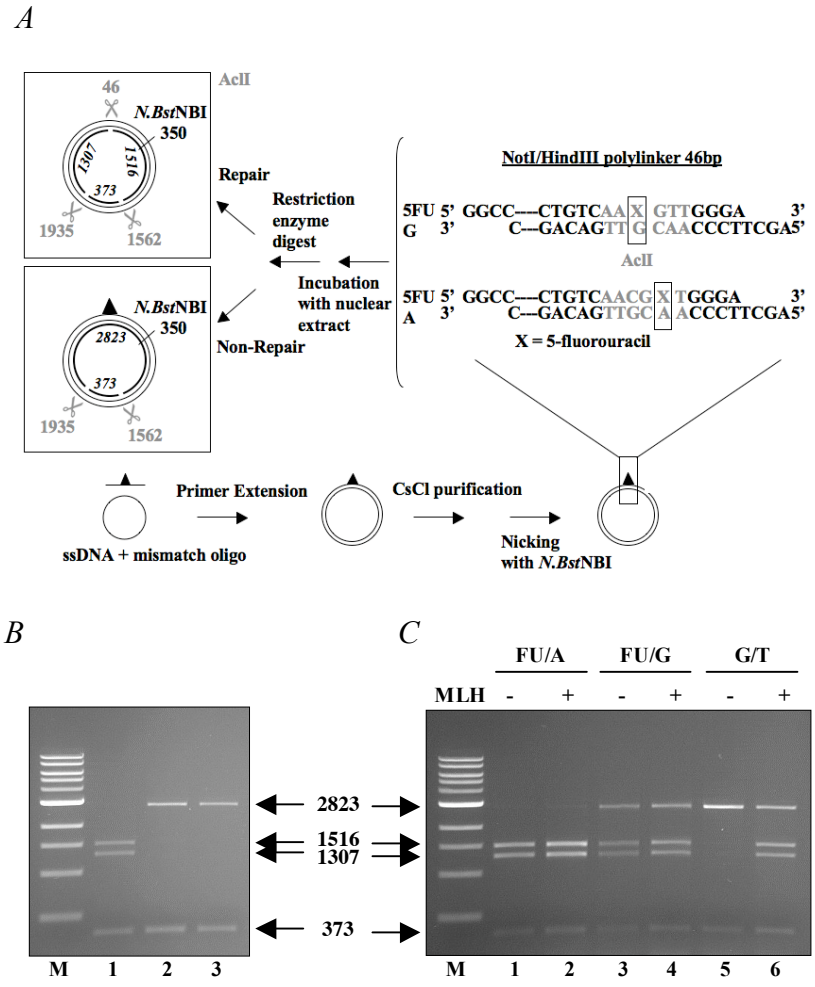
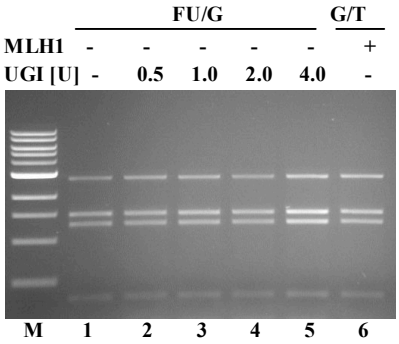


Fischer *et al.*, Figure 2



Fischer *et al.*, Figure 3

A



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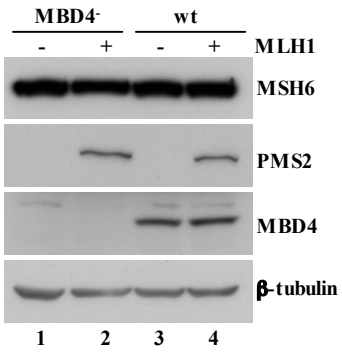
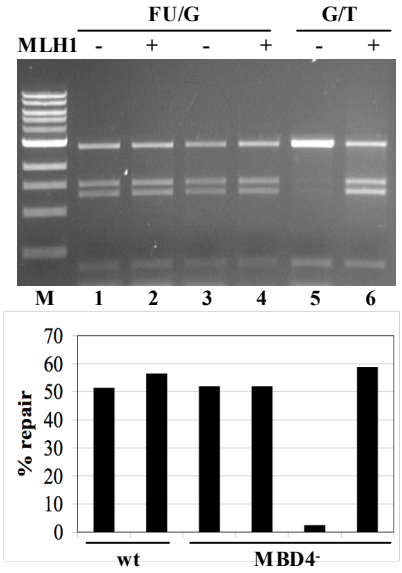
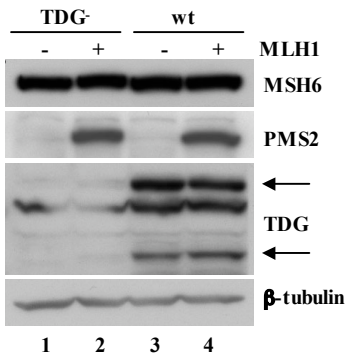
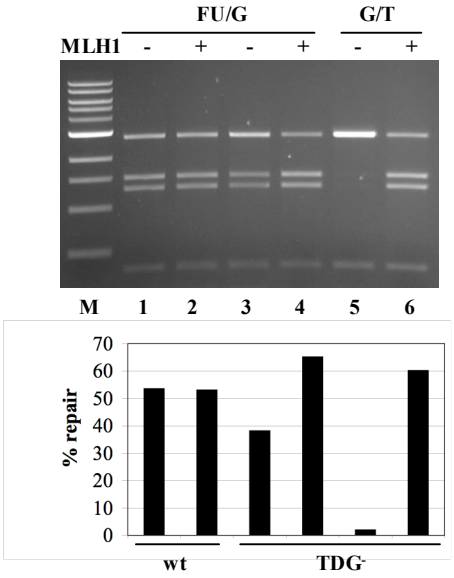


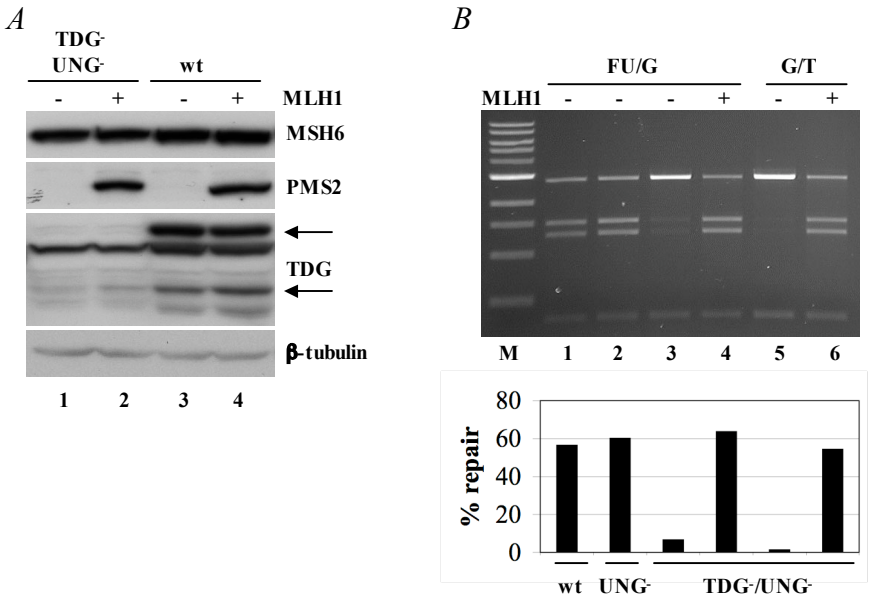


C

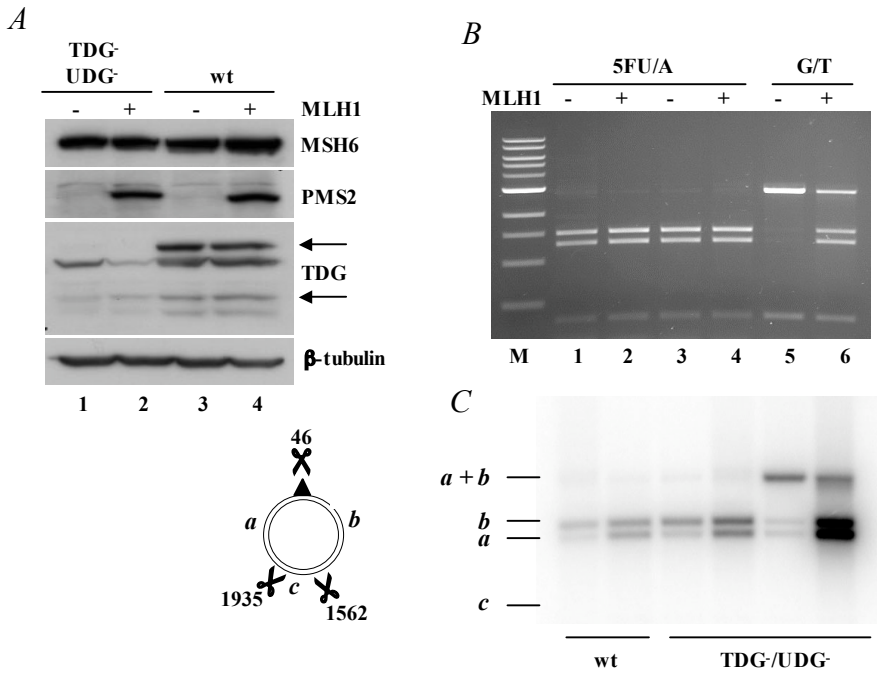




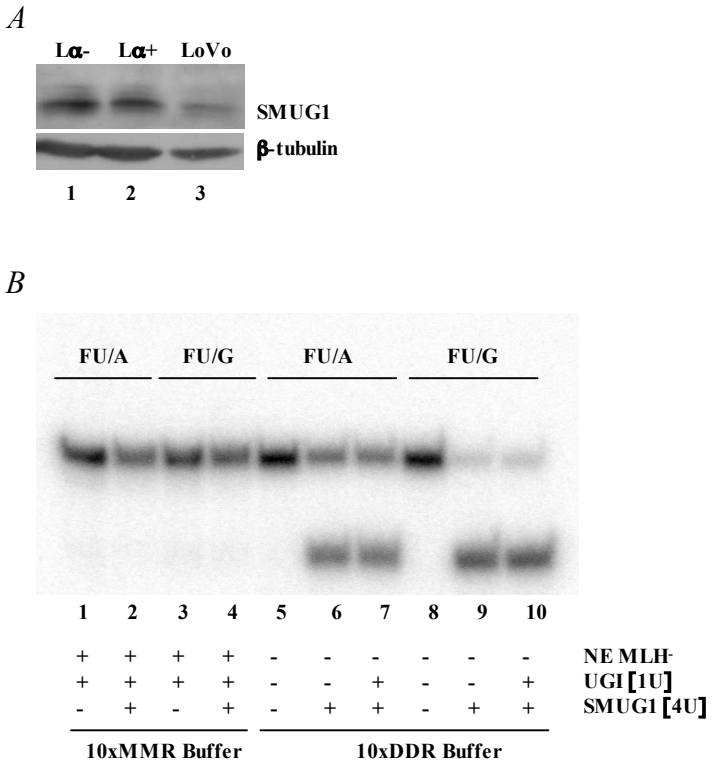
Fischer *et al.*, Figure 4



Fischer et al., Figure 5



Fischer *et al.*, Figure 6



6.2 O^6-methylguanine in the template strand: a futile challenge for the mismatch repair system ?

(manuscript in preparation)

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O^6-methylguanine in the template strand: a futile challenge for human mismatch repair ?

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Running title: Metabolism of O^6-methylguanine in DNA

Keywords: mismatch repair, O^6-methylguanine, heteroduplex substrates

Abstract: 163 words

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Abstract

O^6-methylguanine (^{m}G) is the principal mutagenic and cytotoxic modification induced in DNA by S_N1-methylating agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The exact mechanism by which O^6-methylguanine triggers cell death is unknown. One model suggests that mismatch repair (MMR) recognizes ^{m}G base pairs in the template strand and initiates several iterative excision-resynthesis cycles, finally resulting in persistent discontinuities, such as long single-stranded DNA regions. In an attempt to substantiate this hypothesis, we incubated heteroduplex substrates containing a single ^{m}G lesion with either MMR-proficient or –deficient nuclear extracts from 293TLα cells. Pulse chase experiments in the presence of radiolabeled nucleotides permitted the direct visualization of repair events and putative iterative cycles on the substrates. We could show that ^{m}G-containing mispairs are recognized and processed by MMR, albeit inefficiently, probably resulting in the formation of gapped regions opposite ^{m}G. Unfortunately, the recognition of the substrates by MMR was too weak to allow the detection of possible futile repair cycles and DNA intermediates in the systems we used.

Introduction

By treating DNA with methylating drugs, a plethora of modifications can be created. It is generally believed that agents inducing predominantely N-methylations (S_N2-type agents) such as methylmethanesulfonate (MMS) and dimethyl sulphate (DMS) provoke high chromosome breakage (clastogenic) effects but exhibit low mutagenic potency. On the other hand, agents inducing higher levels of O-methylations (S_N1-type agents) such as *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) are both clastogenic and mutagenic. The critical lesion responsible for the cytotoxicity of S_N1-methylating agents is O^6-methylguanine (^mG**)** [1], which is induced in minor amounts (~7% of the alkylation burden) in the DNA of exposed cells [2]. ^mG is repaired by O^6-methylguanine-DNA methyltransferase (MGMT) [3], and, consequently, cells lacking this repair protein are highly sensitive to O^6-methylating agents compared to MGMT containing cells [4].

In vitro MNU-methylated plasmids exhibited ^mG processing in the form of unscheduled DNA synthesis [5] and this aberrant repair event could be linked to the presence of an intact mismatch repair (MMR) system [6]. Consistent with these results, ^mG -containing oligonucleotides are recognized by the human mismatch repair recognition factor hMutSα in bandshift experiments [7]. The finding that cell lines defective in mismatch correction tolerate DNA-methylation damage induced by S_N1-type drugs [8, 9] confirmed the participation of MMR machinery in ^mG-directed cytotoxicity.

Several models speculating about the mode of action of methylating agents and their ability to induce apoptosis have evolved, but none could be convincingly substantiated so far.

The “futile repair” hypothesis [10] assumes that upon encountering ^mG in the template strand, MMR recognizes the lesion, but fails to repair it since the methylated base does not reside in the newly synthesized strand and thus cannot be addressed by the MMR system. The unsuccessful processing comprises several rounds of excision and re-synthesis steps, finally resulting in aberrant DNA intermediates, which could be converted into harmful double-strand breaks in the following replication round.

Alternatively, the “sliding-clamp signaling” model suggests a new role for MMR as a general DNA damage detector: upon recognition of specific types of DNA damage, MMR proteins directly signal to the apoptotic machinery [11]. Using an isogenic system developed in our laboratory [12], we could recently show that an MNNG-induced G2 arrest in the second cell cycle is absolutely dependent on a functional mismatch repair system and persistence of single-stranded DNA intermediates after the first S-phase could be exclusively detected in MMR-proficient cells [13]. This delayed damage response and preliminary data from electron microscopy studies that reveal single-stranded regions behind yeast and mammalian replication forks (Massimo Lopes, unpublished data) rather support a “futile repair” scenario. Furthermore, Paul Modrich’s lab succeeded in providing evidence for iterative repair cycles opposite ^{m}G *in vitro* [14], endorsing the first model.

But also the “sliding-clamp signaling” model is based on experimental references. Recently, the MMR-dependent recruitment of ATR/ATRIP to ^{m}G/T mismatches *in vitro* and the concomitant phosphorylation of CHK1 was shown, implying a role of MMR proteins as direct sensors of alkylation damage [15]. This is consistent with previous studies, showing that murine MSH2 carrying a missense mutation in the ATPase domain provokes mismatch repair deficiency while retaining apoptotic signaling function in response to DNA-damaging agents. Although the mutated MutSα was resistant to ATP-induced release, the mismatch binding capacity was not affected. From this, the authors concluded that processing of the lesion is not necessary for damage response, thus supporting the “sliding-clamp signaling” model [16]. Similar results were obtained with a dominant missense mutation in the MSH2-MSH6 heterodimer interface of murine MSH6 [17].

In summary, although methylating agents are an integral part of chemotherapeutic regimens for various cancers, the mechanisms of these drugs in eliciting a cytotoxic response in tumour cells are still not fully understood and resistance to chemotherapies remains a major obstacle in cancer treatment.

In an attempt to detect iterative repair opposite ^{m}G and to prove the existence of MMR-dependent single-stranded regions after processing of methylation damage, we incubated heteroduplex substrates containing one single O^6-

methylguanine with nuclear extracts from either MLH1-proficient or –deficient cells, and monitored the repair efficiency with an *in vitro* assay using restriction endonucleases.

Materials and Methods

Substrates, nuclear extracts and in vitro mismatch repair (MMR) assays

The detailed procedure has been described previously [18]. Briefly, heteroduplex DNA substrates containing a G/T, a ^{m}G/T or a ^{m}G/C mismatch within an overlapping *Acl*I/*Sal*I restriction site in the 46 bp polylinker of a pGEM13Zf(+) derivative were constructed by primer extension, using oligonucleotides containing either a G or a ^{m}G as primers and the single-stranded phagemid DNA as template.

The desired supercoiled heteroduplex substrates were purified by caesium chloride centrifugation. The strand discrimination signal was introduced by incubation with *N.Bst*NBI, which specifically nicks the substrates either 3' from the mismatch in the ^{m}G-containing strand (top strand) or 5' from the mismatch in the viral strand (bottom strand) at position 352 of the duplex. The mismatch repair assays were performed in 20 m*M* Tris-HCl pH 7.6, 110 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* glutathione, 50 µg/ml BSA, 0.1 m*M* each dGTP, dCTP, dTTP and dATP, 1.5 m*M* ATP, 100 ng (47.5 fmol) heteroduplex DNA substrate and 50 µg of nuclear extract from 293T-Lα+ ($MLH1_+$), or 293T-Lα- ($MLH1_-$) [12] cells in a total volume of 25 µl. After 30 min incubation at 37°C, the reactions were terminated by the addition of a stop solution and further incubation for 30 min at 37°C (final concentrations: 25 m*M* EDTA, 0.67% SDS, 50 µg/ml proteinase K). The DNA was purified using Qiagen MinElute Reaction Cleanup Kit, digested with the appropriate mismatch-discriminating restriction enzyme and treated with 50 µg/ml RNase A, followed by proteinase K (60 µg/ml) in the presence of 0.2% SDS. The DNA was then precipitated with 0.3 *M* NaOAc pH 5.5 and ethanol, and resuspended in 10 m*M* Tris-HCl. The efficiency of the repair reactions was monitored on 1% TAE agarose (Invitrogen) gels stained with ethidium bromide.

Most of the assays were performed in the presence of 1.7 pmol [α-^{32}P]dATP (10 µCi). To monitor incorporation of the radiolabeled nucleotide, the agarose

gels were vacuum-dried and exposed to a PhosphoScreen (Molecular Dynamics, Inc.).

For pulse chase experiments, MMR reactions were incubated 10 min at 37°C in the presence of 1.7 pmol [α-^{32}P]dATP (10 µCi) and 250 pmol unlabeled dATP (Pulse), followed by addition of 5 nmol of unlabeled dATP (Chase). Samples were usually taken after 10, 20 and 40 min, or as indicated.

Bandshift assays

40 fmol (2 nM) of annealed [γ-^{32}P] 5'-labeled oligonucleotides (see Fig. *1A* for details) were incubated with 100 m*M* KCl, 25 m*M* Hepes-KOH pH 7.5, 1 m*M* DTT, 0.5 m*M* $MgCl_2$, 0.1 m*M* ADP, 10% glycerol, 75 µg/ml BSA and 60 ng poly(dIdC)•poly(dI-dC) competitor (Amersham Pharmacia Biotech) in the presence of different concentrations of purified human MutSα in a final volume of 20 µl. The binding reaction was performed at 37°C for 20 min. Where indicated, oligonucleotides were first incubated for 10 min at 37°C with 0.2U of the human methylguanine methyltransferase MGMT (Sigma) before adding MutSα and incubation for another 15 min at 37°C.

Finally, 10 µl were loaded onto 9x8 cm 5% native polyacrylamide (29:1) gels (ATTO system) eluted with 1x TAE buffer and run 30 min at 19 V/cm. The vacuum-dried gels were exposed to PhosphoScreens (Molecular Dynamics, Inc.).

MGMT treatment of heteroduplex ^{m}G substrates

MGMT treatment of ^{m}G-containing heteroduplex substrates was performed in 25 m*M* Hepes-KOH pH 7.5, 1 m*M* DTT, and 75 µg/ml BSA in the presence of 0.2U of the human methylguanine methyltransferase MGMT (Sigma) in a final volume of 20 µl. After 15 min incubation at 37°C, reactions were supplemented with appropriate amounts of BSA and restriction buffer and subsequently digested with *Acl*I or *Sal*I/*Ban*I endonucleases, respectively.

Dot Blot

DNA was spotted under vacuum onto a Zeta-membrane (Zeta-Probe GT Genomics, Biorad), incubated in 2x SSC for 5 min and cross-linked to the

membrane with UV Stratalinker 1800 (Stratagene). The 6x SSC-wetted membrane was pre-hybridized for 2 hours at 45°C with hybrisol I (Chemicon International) in rotating glass tubes.

Subsequently, the membrane was incubated overnight with 10 pmol of a [γ-^{32}P] 5'-labeled probe (5' CAA TTC TCA AGC TTC CCA ACG TCG ACA GAC GTC TGG 3') in hybrisol I solution. Finally, the dot blot was washed consecutively with 2x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1% SDS (15 min for each washing step) and exposed to a PhosphoScreen (Molecular Dynamics, Inc.).

As a positive control, the [γ-^{32}P] 5'-labeled probe was annealed to 10 pmol of a complementary oligonucleotide, and 100 ng of non-complementary closed-circular single-stranded DNA served as a negative control.

Results

Recognition of ^{m}G-containing mispairs by human MutSα

To confirm the ability of the human mismatch recognition factor MutSα to detect and bind to O^6-methylguanine (^{m}G) in double-stranded DNA [7], we performed bandshift experiments with oligonucleotides harboring different mispairs (*Fig. 1A*).

In contrast to G/T mismatches that are efficiently recognized and bound by MutSα, ^{m}G/T and ^{m}G/C mispairs, at least in our sequence context, were no better substrates than a G/C homoduplex control (*Fig. 1B*). Observations with different oligonucleotides made by *Hsieh et al.* [15], where the ^{m}G/T and G/T substrates were bound with comparable efficiency suggested that the binding of MutSα to ^{m}G/T may be affected by flanking sequences. However, in our hands, no appreciable binding could be detected even in a different sequence context (data not shown). As the sequences used in our experiment were bound as strongly as the G/T substrate by yeast MutSα, we concluded that the human mismatch binding protein recognizes the methylated G/T and G/C substrates with low efficiency. The quantification of the bandshifts shown in *Fig. 1B* is depicted in *Fig. 1C*. In order to ensure the integrity of our substrates, we pretreated our ^{m}G/T oligonucleotides with human methylguanine methyltransferase (MGMT) prior to incubation with MutSα,

thereby restoring a G/T mismatch that is strongly bound by human MutSα (*Fig. 1D*). In summary, human MutSα recognizes both ^{m}G/T and ^{m}G/C mispairs equally weakly and to a similar extent as homoduplex DNA in bandshift assays.

Endonuclease susceptibility of ^{m}G-containing mispairs in heteroduplex DNA substrates

To study mismatch repair-dependent processing of ^{m}G in more detail *in vitro*, we constructed heteroduplex DNA substrates containing a ^{m}G either opposite C or T. The former mispair resides within an *Acl*I restriction site, the latter lies within an overlapping *Acl*I/*Sal*I sequence (see *Fig. 3A*). Because the MMR assay is based on conversion of the restriction-endonuclease resistant, mismatch-containing vector, to a cleavage-susceptible form, we first had to test whether our ^{m}G/C and ^{m}G/T substrates are indeed resistant to the respective endonucleases. As shown in *Fig. 2*, ^{m}G/C mispairs are indeed *Acl*I-resistant (*lane 2*), while a considerable portion of ^{m}G/T mismatches, although resistant to *Sal*I (*lane 8*), can be cleaved by *Acl*I (*lane 5*). The presence of ^{m}G was confirmed by treating the ^{m}G/T and ^{m}G/C substrates with MGMT (*lanes 3, 6 and 9*), thereby restoring G/T and G/C base pairs, respectively. G/T and G/C substrates served as controls (*lanes 1, 4 and 7*). These results confirm that ^{m}G/T resembles more closely a Watson-Crick base pair than ^{m}G/C [19] and provide an explanation for the observed *Acl*I sensitivity of ^{m}G/T substrates.

Processing of ^{m}G-containing base pairs in nuclear extracts of human cells

With the aid of an *in vitro* MMR assay (*Fig. 3A*), we addressed MMR-dependent processing of ^{m}G residues located either in the template (unnicked, circular) or the newly synthesized (*N.Bst*NBI nicked) strand. Upon incubation of heteroduplex substrates containing single G/T, ^{m}G/T or ^{m}G/C mismatches with nuclear extracts from MLH1-proficient (+), or MLH1-deficient (-) 293T-Lα cells, we could examine the repair process in some detail. As

shown in *Fig. 3B*, the MMR machinery clearly recognized and repaired ^{m}G lesions from the nicked strand, although substantially less efficiently than G/T mismatches (*left panel, compare lane 2 with lanes 4 and 6)* [7], consistent with our bandshift results. A different situation arises when the MMR system encounters a ^{m}G in the template strand. Since such a lesion cannot be removed, we expected it to be left unrepaired in our MMR assay. Indeed, no indication of repair of heteroduplex substrates containing ^{m}G in the unnicked strand could be observed (*Fig. 3B, right panel, lanes 4 and 6*).

Recently, Modrich and colleagues [20, 21] reported that repair of the 5'-G/T heteroduplex in their reconstituted system with purified proteins occurred efficiently in the absence of human MutLα, although MutSα and EXOI were required for the reaction. We agree with these findings, since also in our hands 5'-G/T shows significant background repair in the absence of MutLα, which can be abolished by performing MMR assays with MSH2-deficient LoVo nuclear extract. Furthermore, aphidicolin inhibited both background repair in MLH1-deficient and MMR-specific repair in MLH1-proficient nuclear extract, thus supporting the idea that MutSα and probably both EXOI and polymerase δ are responsible for the observed 5'$\rightarrow$3' activity (data not shown). It is however noteworthy that the extent of background repair was dependent on the batch of substrate and/or the nuclear extract preparation, since this phenomenon was not reproducible (*compare Fig. 3, right panel, lanes 1 and 2 with Fig. 5D, lanes 5 and 6*).

In summary, MMR machinery recognizes and repairs ^{m}G, albeit with low efficiency, provided that the lesion resides in the nick-containing strand.

Futile repair opposite ^{m}G lesions

In an attempt to contribute to the longstanding hypothesis of futile repair events opposite a template ^{m}G, we set out to take advantage of our *in vitro* MMR assay and performed pulse chase experiments. The idea behind these experiments was to incubate our ^{m}G substrate with nuclear extracts in the presence of radiolabeled dATP, which is supposed to be incorporated mainly in the excision tracts formed upon repair attempts. By the addition of excess non-radioactive dATP after some minutes, we hoped to detect a decrease in

the amount of incorporated radioactivity due to the proposed futile cycling opposite ^{m}G (*Fig. 4A*). In contrast to recently published data [14], we could never observe a ^{m}G-provoked and MMR-dependent decrease in radioactivity after cold-chasing the reaction. Moreover and unexpectedly, the most pronounced effect could be seen with the G/T substrate (*Fig. 4B and 4E*). Although ^{m}G/T substrates revealed a MMR-specific decrease of radioactivity of ~20% (*Fig. 4D and 4E*), ^{m}G/C substrates did not show any difference between MMR-deficient and –proficient backgrounds (*Fig. 4C and 4E*).

In another approach we separated MMR reactions containing radioactive dATP by means of Thin Layer Chromatography (TLC) and tried to detect the side-product dAMP, which should arise during the excision step of putative futile repair cycles (data not shown). Unfortunately, dATP was already hydrolyzed by solely adding nuclear extract, thereby masking MMR-dependent hydrolysis.

Thus, we cannot confirm the futile cycling scenario opposite ^{m}G, but rather observe a general decrease of radioactivity within our DNA substrates over time.

Incorporation of C or T opposite ^{m}G remains ambiguous

Which base is chosen to complement ^{m}G in the template strand ? To find an answer to this question we tried to exploit our mismatch-discriminating restriction enzymes *Acl*I and *Sal*I once more. After incubation of ^{m}G/T substrates with a nick in the T-containing strand and in the presence of all MMR proteins, three different situations opposite ^{m}G came into play: incorporation of either T or C, or formation of a gap due to unsuccessful processing. To leave aside the latter and by assuming that either ^{m}G/T or ^{m}G/C are formed, we theoretically end up with G/T and G/C base pairs exhibiting characteristic endonuclease susceptibility after MGMT treatment (*Fig. 5A*).

However, the outcome of the experiments was not clear. Our results suggested that both ^{m}G/T and ^{m}G/C maintained (or restored) the original base pair conformation: ^{m}G/T showed *Acl*I and *Sal*I resistance upon MGMT treatment, implying the existence of a G/T mismatch (*Fig. 5B and 5C*), and ^{m}G/C turned out to be *Acl*I-sensitive after incubation with MGMT, a proof for

the presence of a G/C base pair and an argument against the formation of gaps (*Fig. 5D*). The fact that only ~10% of the molecules retained the *Acl*I-resistant phenotype (fragments indicated by an asterisk) in a MMR-independent way further substantiates this conclusion. On the other hand, we cannot fully exclude the possibility of gaps arising, especially for ^{m}G/T mismatches (*see also Fig. 6A*).

No gaps are detected upon repair events opposite ^{m}G

Since ^{m}G/T substrates are clearly processed in the presence of nuclear extract, thereby becoming considerably *Acl*I-resistant (*Fig. 6A*), we wanted to have a closer look at possible gaps arising during the repair trial. To this end we spotted purified MMR reactions on a membrane and hybridized a radiolabeled probe to the samples. Although MMR on a G/T substrate worked properly in this approach (*Fig. 6B, right panel*), no signal could be detected in any of the tested substrates (*Fig. 6B, left panel, lanes 1-8*).

Fill-in assays with Klenow polymerase 3'→5' Exo$^-$ (deficient in both 3'→5' and 5'→3' exonucleolytic activities) after MMR reactions could not show MMR-dependent gap formation (data not shown, see **Discussion**).

Discussion

A functional mismatch repair system (MMR) in combination with ^{m}G moieties in DNA yields a cytotoxic composite causing cells to arrest in the G2/M phase of the second cell cycle [13]. Although we and others provided pieces of evidence for the formation of processed DNA intermediates due to repeated but unsuccessful attempts of MMR to correct the lesion, the exact mechanism of the observed cytotoxicity remains speculative.

To confirm the hypothesis predicting "futile repair" events opposite ^{m}G in the template strand thereby leading to single-stranded DNA intermediates, we used an *in vitro* MMR system with heteroduplex substrates containing one single O^6-methylguanine in the parental (unnicked) strand and nuclear extracts from the isogenic 293TLα cell line, being either MLH1-proficient or MLH1-deficient [12]. We established an assay to detect incorporation of radiolabeled nucleotides and repair efficiency on our substrates (*Fig. 3B*), but

neither pulse-chase experiments (*Fig. 4*) nor thin layer chromatography to monitor the excised dAMP (*data not shown*) could prove the futile repair scenario in our hands. However, we cannot exclude the existence of the latter phenomenon, for reasons that will be briefly discussed.

As shown in bandshift and MMR assays, our ^{m}G-containing substrates were poorly recognized by hMutSα using two different sets of oligonucleotides (*Fig. 1B, 3B and data not shown*). This has been ascribed to a certain sequence-dependence of MMR factors, and other groups indeed reported that hMutSα bound ^{m}G/T mismatches as well as G/T mismatches ([15]; Paul Modrich, personal communication) in a specific sequence environment. Interestingly, yeast MutSα efficiently recognized ^{m}G/T mismatches in our sequence context (*data not shown*).

To monitor radioactive incorporation and MMR efficiency, we've used standard agarose gels for our assays. Although eminently suitable for standard MMR assays, agarose gels may lack the resolution necessary to detect the small differences occurring in these reactions. Moreover, assuming that the inherently weak recognition of our ^{m}G-substrates will not result in a large amount of the desired DNA intermediates, the detection of these aberrant structures might need more sensitive read-outs. Indeed, working on the same topic, Paul Modrich's laboratory recently demonstrated futile repair events opposite ^{m}G with the aid of alkaline agarose and polyacrylamide gels [14].

The elegant idea to show *in vitro* futile repair cycles by iteratively excised nucleotides in the form of radiolabeled dAMP with thin layer chromatography failed due to the fact that dATP is hydrolyzed to dAMP in the mere presence of nuclear extract, thus masking the effect of ^{m}G-induced hydrolysis (*data not shown*).

To visualize the hypothesized single-stranded gaps presumably formed upon ^{m}G processing, we performed fill-in assays after MMR reactions by using Klenow polymerase 3'→5' Exo$^-$. The residual 5'→3' polymerase activity was expected to fill the presumable gaps and replicate across ^{m}G [22] without exonucleolytic side reactions. Unfortunately, we encountered a major obstacle in the purification step and we fear that the commercially available columns as

parts of DNA purification kits are not suitable for the recovery of special DNA intermediates. We are currently trying to optimize the protocol by phenol-chloroform purification of the DNA.

Another approach, called Dot Blot, was tested to gain more insight into gapped structures by spotting DNA on a special membrane after MMR reaction on ^{m}G-substrates, followed by hybridization with a radiolabeled probe that is complementary to the ^{m}G-containing strand (*Fig. 6B*). We could not detect a signal so far, and this could be for several reasons: 1) the amount of generated gaps due to futile repair cycles is too low to be detected, or, in other words, the amount of DNA has to be increased to see an effect; 2) the size of the gap is shorter than our probe and thus cannot be detected; 3) the gaps within the substrates are not accessible for the probe; 4) there are no gaps. Since the controls worked very nicely, we shall try to optimize the conditions in order to find an answer.

Since tolerant cells remain sensitive to the mutagenic effect of alkylating agents, questions about the replication across ^{m}G arise. Several studies *in vitro* [23-25] and *in vivo* [26-28] demonstrated a preferential, albeit quite inefficient, incorporation of dTMP opposite a ^{m}G template, for this lesion blocks synthesis by prokaryotic and eukaryotic DNA polymerases in general. This applies also to the repair polymerase β [22], although the latter can catalyze the ^{m}G bypass *in vitro*, provided that the lesion resides in single- or short nucleotide gapped substrates [29]. DNA polymerase α, required for lagging strand synthesis, is strongly blocked one base before ^{m}G [30]. The human and yeast translesion polymerases η (also called Rad30 in yeast) are very efficient in bypassing ^{m}G and inserting nearly equally well a C or a T opposite the lesion ([19], reviewed in [31]). The replicative human and yeast polymerase δ can bypass ^{m}G as well, but this lesion presents a strong block and favors the error-prone incorporation of a T [19]. Another translesion polymerase, the yeast polymerase ζ, was shown to act as an extender from the nucleotides inserted opposite ^{m}G by polymerase δ [32]. But which nucleotide is incorporated in the presence of the full complement of polymerases ? In an attempt to find an answer to this question, we took advantage of the different endonuclease sensitivities of our ^{m}G substrates

(*Fig. 5*). But once more: the weak recognition of our substrates by the MMR system failed to yield a conclusive result. $^{m}G/T_b$ clearly became *Acl*I- and *Sal*I-resistant *(Fig. 5B, left panel, lanes 1-4; middle panel, lanes 1-4)* after the MMR reaction and MGMT treatment, implying the formation of a G/T mismatch. The weak persistence of the two repair fragments *(left panel, lane 4)* can be explained by the spontaneous generation of unspecific nicks in the top strand and concomitant repair by the MMR to restore an *Acl*I-susceptible A/T site. On the other hand, $^{m}G/C_b$ became *Acl*I-sensitive after MMR reaction and MGMT treatment, suggesting the maintenance of the original base-pair *(Fig. 5, right panel, lanes 1-4)*. Three conclusions can be drawn: 1) the complex interplay between several different polymerases incorporating distinct nucleotides concealed the outcome, 2) our system is not sensitive enough to reveal the MMR-directed incorporation of the favored nucleotide opposite ^{m}G due to the weak recognition of the lesion, or 3) the arising gaps predominate and cannot be detected by this approach.

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Legends to Figures

Fig. 1 Recognition of ^{m}G-containing mispairs by MutSα in bandshift assays.
A, Oligonucleotide substrates G/T, ^{m}G/C, ^{m}G/T or G/C. *B,* Binding of MutSα to ^{m}G/T and ^{m}G/C mismatches. The [γ-^{32}P] 5'-labeled oligonucleotides (G/C, G/T, ^{m}G/C, ^{m}G/T) were incubated with increasing concentrations of purified human MutSα, as indicated. *C* Quantification of the gels. The shifted bands were quantified using the ImageQuant TL (Amersham Biosciences) software. *D,* Methylguanine methyltransferase (MGMT) efficiently converts ^{m}G to G. G/T and ^{m}G/T oligonucleotides were incubated with 103.5 nM MutSα in the absence (*lanes 1 and 2*) or presence (*lanes 3and 4*) of MGMT (0.2U). The panels *B* and *D* are autoradiograms of 5% native polyacrylamide gels.

Fig. 2 Susceptibility of ^{m}G-containing heteroduplex substrates to cleavage with the endonucleases *Acl*I and *Sal*I.
The heteroduplex DNA substrates (see *Materials and Methods* and *Fig. 3*) containing a ^{m}G/C mismatch were tested for *Acl*I-sensitivity only (*lanes 2 and 3*), whereas the ^{m}G/T substrates were tested with both endonucleases, *Acl*I

and *Sal*I (*lanes 5 and 6, 8 and 9*) in the presence (*lanes 3, 6 and 9*) or absence of 0.2 U MGMT. The resulting fragments are labeled *a-g*.

Fig. 3 Processing of ^{m}G-containing base pairs in nuclear extracts of human cells.

A, Schematic representation of the construction of the ^{m}G-containing DNA substrates and of the *in vitro* MMR assay. *B, In vitro* MMR assays performed with G/T, ^{m}G/T or ^{m}G/C substrates. The 1516 bp and 1307 bp *Acl*I restriction fragments indicate repair of G/Tt, ^{m}G/Tt, ^{m}G/Ct and ^{m}G/Cb, the 422 bp and 141 bp (not visible) restriction fragments generated by *Sal*I/*Ban*I monitor repair of G/Tb and ^{m}G/Tb. The reactions were carried out in nuclear extracts of MMR-proficient (+), or MMR-deficient (-) 293T-Lα cells in the presence of [α-^{32}P]dATP, separated on a 1% TAE agarose gel (*upper panel*) and finally exposed to a PhosphoScreen (*lower panel*). t, substrate containing a specific nick in the complementary ("top") strand; b, substrate containing a specific nick in the viral ("bottom") strand.

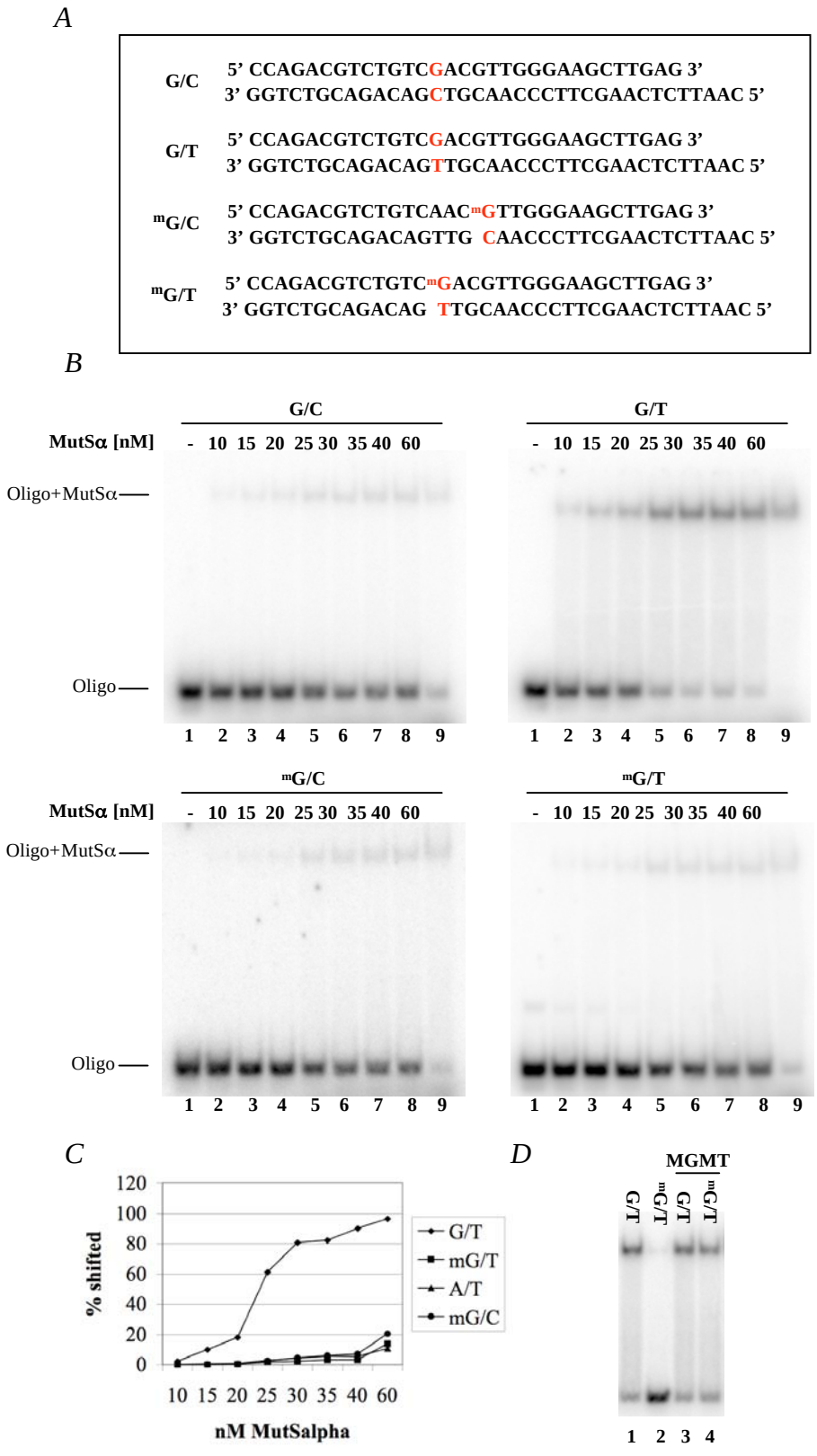
Fig. 4 Pulse chase experiments fail to provide evidence of futile cycling.

A, Experimental design of the pulse chase experiments. NE: nuclear extract of MMR-proficient 293T-Lα cells. *B,* Pulse chase with the G/T substrate. The heteroduplex DNA was incubated with either MMR-deficient (Lα-) or MMR-proficient (Lα+) nuclear extract in the presence of [α-^{32}P]dATP. Aliquots were withdrawn after 10, 20 and 40 minutes. After the10 minute pulse, an excess of cold dATP was added to the reaction (chase), indicated by black arrows. The 1% TAE agarose gel showing the *Acl*I digest of the repair reactions (*upper panel*) was finally vacuum-dried and exposed to a PhosphoScreen (*lower panel*). *C,* Pulse chase with the ^{m}G/C substrate. The procedure was as described in *B*. *D,* Pulse chase with the ^{m}G/T substrate. The procedure was as described in B, with the exception that aliquots were withdrawn after 10, 15, 25 and 40 minutes. *E,* Quantification of the pulse chase experiments. For each time point, the total incorporation of radiolabeled dATP within the substrate was quantified. The level of radioactive incorporation after 10 minutes (pulse) was taken as 100%.

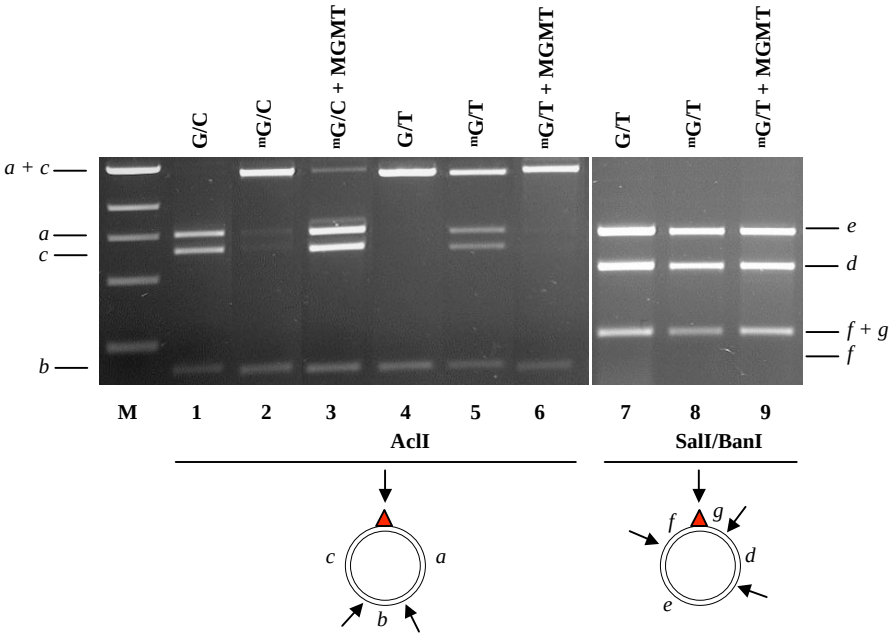
Fig. 5 Processing of mispairs containing ^{m}G in the template strand.
A, Schematic representation of the possible outcomes of MMR-dependent *in vitro* processing and subsequent MGMT treatment of substrates containing the ^{m}G residue in the continuous strand, as measured by susceptibility to the endonucleases *Acl*I and *Sal*I after. ✗, endonuclease-resistant; ✓, endonuclease-sensitive. *B, C,* The ^{m}G/T substrate was incubated with either MMR-deficient (-) or MMR-proficient (+) nuclear extracts, followed by MGMT treatment (**n**: no MGMT; **y**: MGMT-treated) and restriction digest with the appropriate endonucleases *Acl*I (*B, lanes 1-4*) or *Sal*I/*Ban*I (*C, lanes 1-4)*). The repair efficiency was compared to that of the G/T substrate (*B and C, lanes 5 and 6*). The upper panels show agarose gels, which were vacuum-dried and exposed to PhosphoScreens (*middle panels*). On the bottom, formation of fragments that are indicative of repair was quantified (compare *Fig. 3*). *D,* Procedure identical to *B* and *C*, exception that the ^{m}G/C substrate was used. The G/T substrate containing the nick in the viral strand meaning the restoration of the *Sal*I restriction site upon repair was used as the positive control (*lanes 5 and 6*).

Fig. 6 Nuclear extracts of 293T-Lα cells processes ^{m}G-containing heteroduplexes independently of their MMR status.
*A, Acl*I susceptibility of the ^{m}G/T substrate after incubation with the endonuclease alone (*left*) or with nuclear extract from MMR-deficient (-) or MMR-proficient (+) 293T-Lα cells (*centre*). *Acl*I fragments arising due to cleavage at the mismatch site are marked with an asterisk. Quantification of the *Acl*I cleavage is depicted on the right. *B, Left panel:* Dot Blot with ^{m}G/Tb *(lanes 1 and 2)*, ^{m}G/Cb *(lanes 3 and 4)*, G/Tb *(lanes 5 and 6)* and G/Tt *(lanes 7 and 8)* substrates, which were incubated with nuclear extract from MMR-deficient (-) or MMR-proficient (+) 293T-Lα cells, subsequently spotted onto a Zeta-membrane and hybridized with a radiolabeled probe complementary to the O^6-methylguanine- *(lanes 1-4)* or guanine-containing *(lanes 5-8)* strands, respectively. A complementary 31-mer oligonucleotide served as the positive control *(Positive C.)*, non-complementary single-stranded DNA was used as the negative control *(Negative C)*. *Right panel:* MMR reaction with a G/Tt substrate was used to check the repair efficiency of the experiment.

Fischer *et al.*, Figure 1

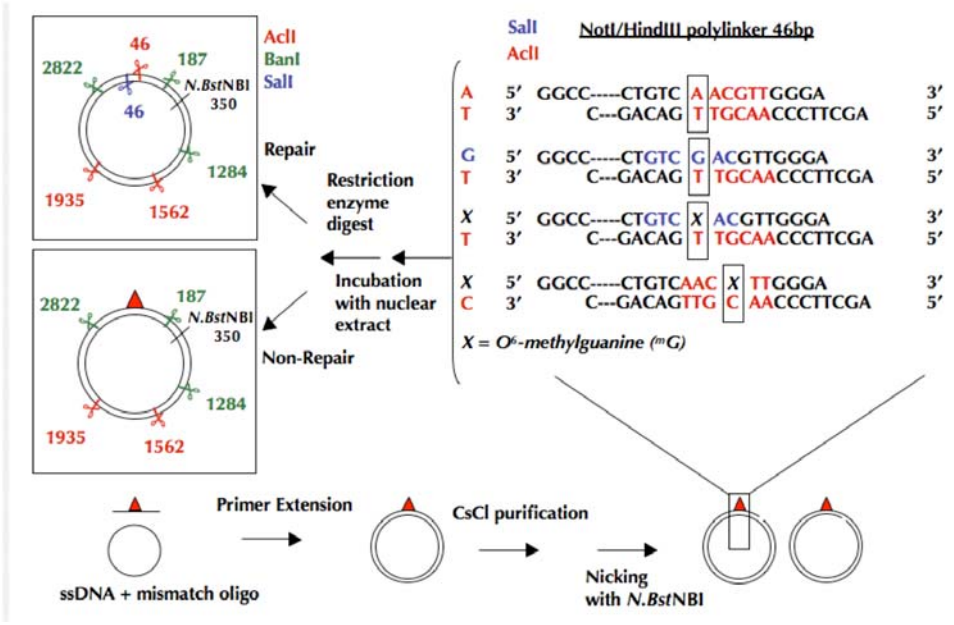


Fischer *et al.*, Figure 2



Fischer *et al.*, Figure 3

A



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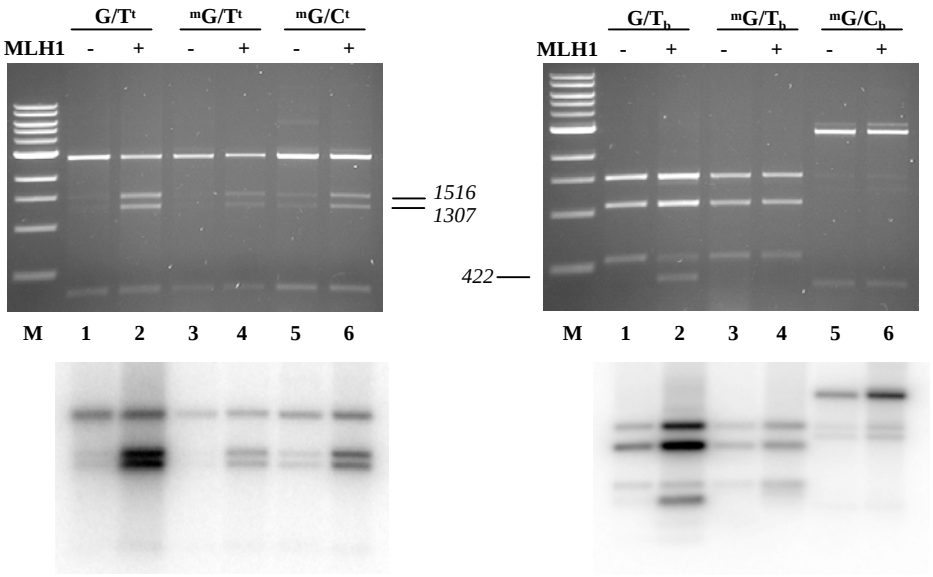
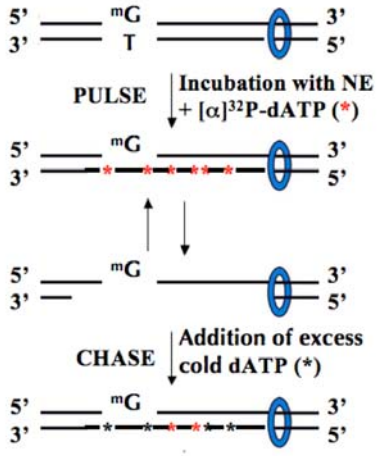


Fischer *et al.*, Figure 4

A



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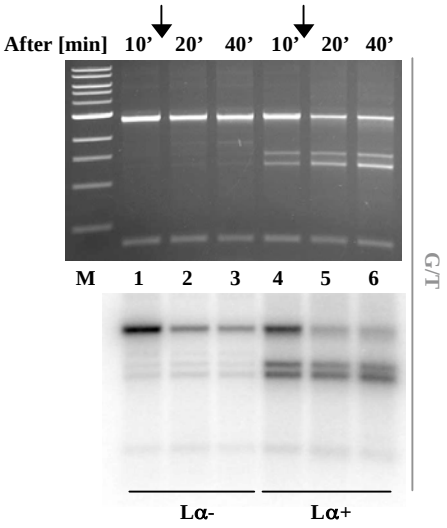

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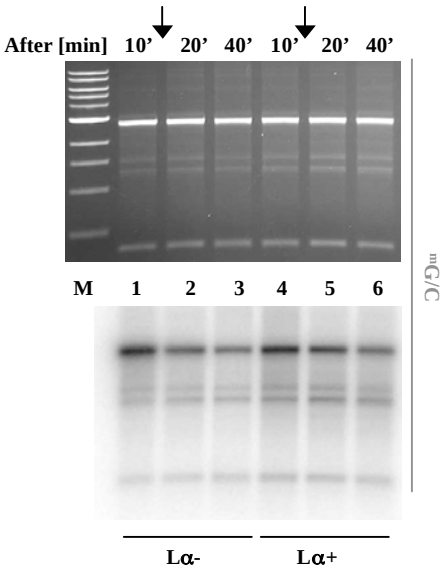

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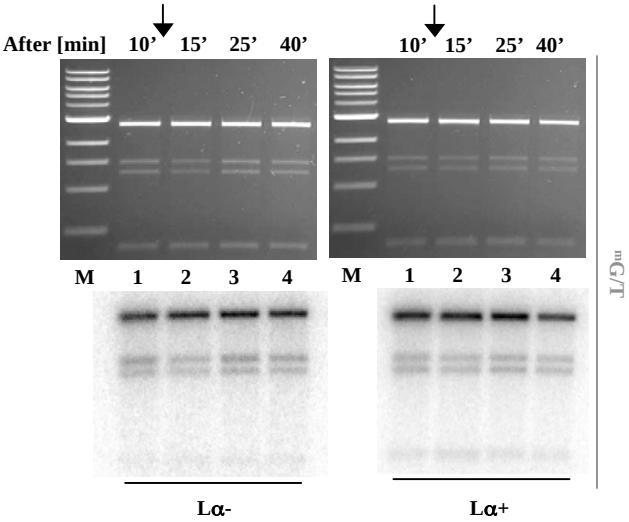
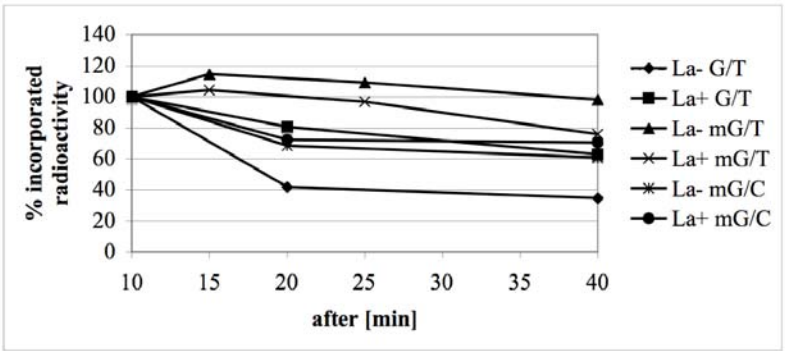
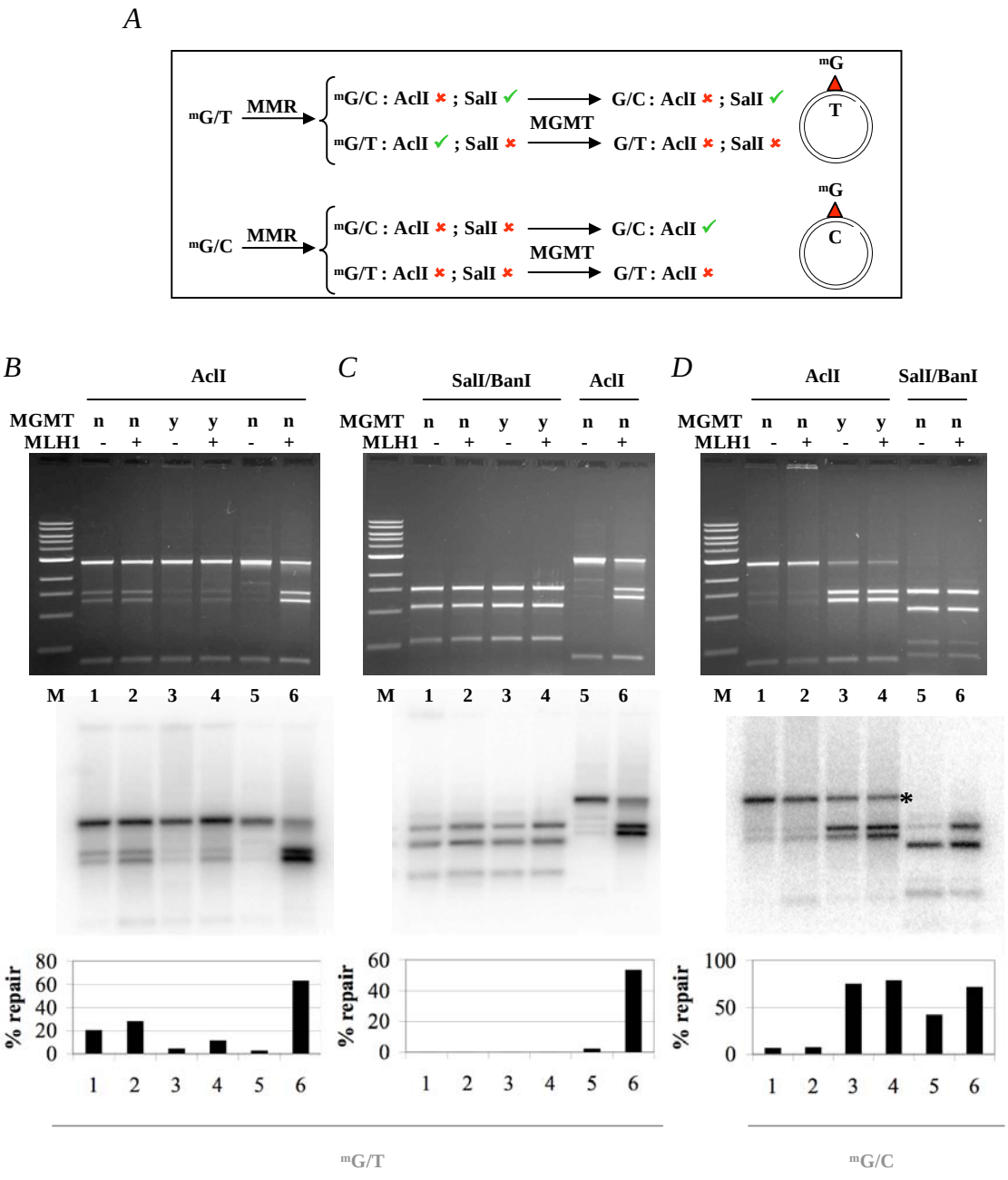


E

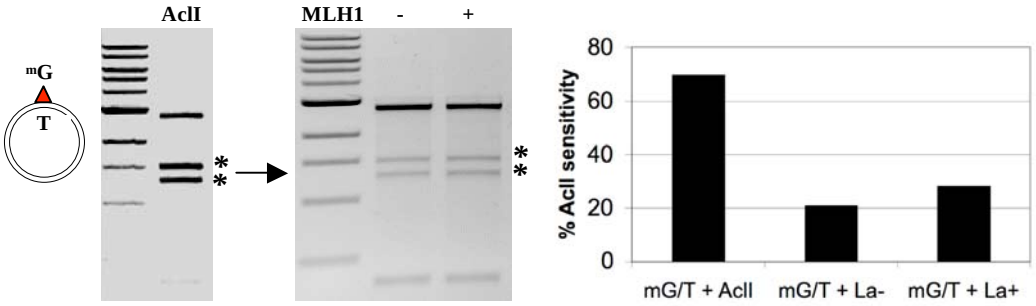


Fischer *et al.*, Figure 5



Fischer *et al.*, Figure 6

A



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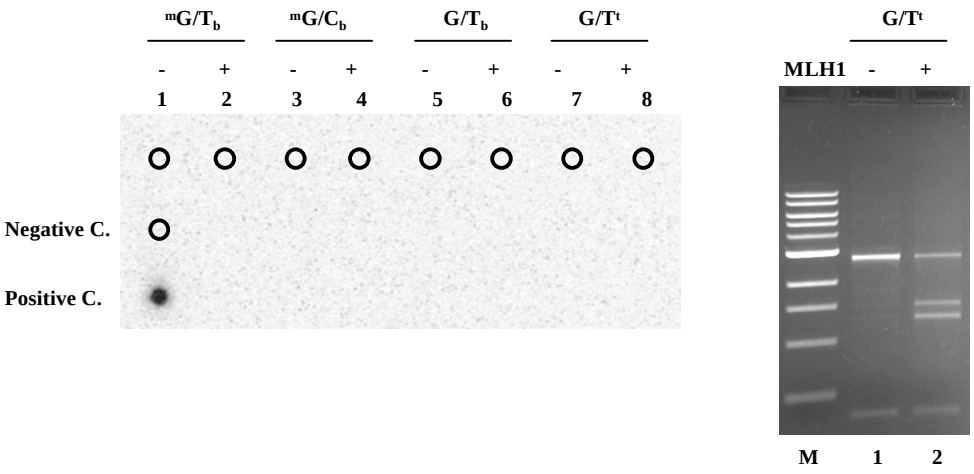

6.3 Characterization of the "mismatch repairosome" and its role in the processing of modified nucleosides *in vitro*

(Methods in Enzymology, 2006;408:285-303)

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[18] Characterization of the "Mismatch Repairosome" and Its Role in the Processing of Modified Nucleosides *In Vitro*

By KATJA BAERENFALLER, FRANZISKA FISCHER, and JOSEF JIRICNY

Abstract

The process of postreplicative mismatch repair (MMR) increases the fidelity of DNA replication by eliminating biosynthetic errors from newly synthesized DNA. In addition, MMR proteins are also involved in the processing of intermediates of mitotic and meiotic recombination and, in mammalian cells, play a role in DNA damage signaling. As mismatches cannot be induced in the DNA of living cells, the study of the molecular transactions during MMR is restricted to *in vitro* systems. This chapter describes the construction of heteroduplex substrates that can be used for DNA affinity purification of MMR protein complexes and for the study of the role of eukaryotic MMR proteins in the processing of modified nucleosides.

Introduction

The mismatch repair (MMR) system can increase the fidelity of DNA replication by up to three orders of magnitude. It accomplishes this task by recognizing base/base mismatches and strand misalignments (insertion/deletion loops, IDLs) that have escaped the proofreading function of the replicative polymerase and by catalyzing a regional degradation of the mismatch-containing DNA strand, which results in removal of the non-Watson–Crick structures. Resynthesis of the degraded region restores the integrity of the replicated duplex (Modrich and Lahue, 1996). As it is the newly synthesized DNA strand that carries the erroneous genetic information, the correction process has to be directed to this strand (Modrich, 1997). In eukaryotes, the directionality of MMR is made possible by an intimate coupling of the replication and repair processes, mediated most likely by proliferating cell nuclear antigen, the processivity factor of DNA polymerases (Jiricny and Marra, 2003). Because the eukaryotic MMR system lacks detectable endonuclease activity (Holmes *et al.*, 1990; Thomas *et al.*, 1991), degradation of the error-containing strand must begin at a preexisting strand discontinuity, such as either end of an Okazaki fragment or the 3′ terminus of the primer strand (Modrich, 1997).

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The mammalian MMR reaction works extremely well in cell-free systems. To date, the tested substrates have been heteroduplexes based on fd phage (Holmes *et al.*, 1990) or M13 (Thomas *et al.*, 1991), which were obtained by annealing of a linearized double-stranded DNA of one phage with the single-stranded circular (viral) form of another phage that differed from the first in a defined position. Isolation of circular double-stranded molecules yielded a substrate containing a mismatch at a defined site, together with a nick a given distance 5′ or 3′ from the mispair. The disadvantage of this approach was the large size of the DNA used (fd phage and M13 are 6–7 kb in length), which limits the yields, and the fact that the nick could only be positioned in the complementary strand.

We are interested primarily in isolating the ''mismatch repairosome'' by DNA affinity purification, which requires considerable amounts of heteroduplex DNA. To this end, we have modified the construction method of the heteroduplex substrate. We make use of phagemid DNA, which is ~3 kb in size and can be isolated in large amounts in both single- and double-stranded forms. Our second field of study is concerned with the investigation of the role of MMR proteins in the processing of modified nucleosides. In this experimental system we need to introduce site-specific modifications into either strand of the heteroduplex. To achieve this goal, we have modified the pGEM-13Zf(+) vector, such that it carries only a single recognition site for *N.Bst*NBI, an enzyme, which cleaves only a single strand of this asymmetric sequence and thus introduces only a single nick into our duplex DNA molecules (see also Wang and Hays, 2001, 2002).

The following paragraphs describe the preparation of both these substrates and include also some pilot experiments with extracts of human cells.

Construction of Substrates Containing Insertion/Deletion Loops

The heteroduplex substrates containing an insertion/deletion loop (IDL) of a single extrahelical nucleotide are constructed from two different plasmids, one of which is 1 bp longer than the other. To produce these, two oligonucleotides, 5′-GGCCGCTCCATGCAAGCCTAG**A**GATCTCCCTCAAGTAGTA-3′ and 5′-GGCCGCTCCATGCAAGCCTAGGATCTCCCTCAAGTAGTA-3′, differing in one position (denoted in bold), are cloned between the *Hin*dIII and the *Not*I restriction sites of the phagemid pGem-13Zf(+). The first of the resulting plasmids, pGem_IDLinker40, contains a unique *Bgl*II restriction site, whereas the second, pGem_IDLinker39, contains a unique *Avr*II site. By linearizing the two

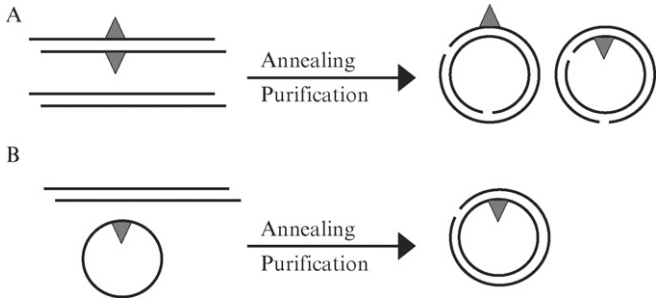

FIG. 1. Schematic representation of the production of (+/−) substrates (A) and 3′ and 5′ substrates (B).

double-stranded DNAs with different restriction enzymes, denaturing and reannealing, a mixture of two circular heteroduplexes can be obtained, 50% of which contain an extrahelical adenosine in one strand and the other 50% an extrahelical thymidine in the other strand (Fig. 1A). This substrate, which is referred to as the (+/−) substrate, is simple to obtain in large amounts and is used for DNA affinity purifications. The second heteroduplex can be obtained by annealing a linearized double-stranded DNA of one phagemid with the circular single-stranded DNA of the other (Fig. 1B). In this way, heteroduplexes containing either a single extrahelical thymidine in the viral strand or a single extrahelical adenosine in the complementary strand can be obtained. By using different phagemids, heteroduplexes carrying any type of base/base mismatch or any size IDL can be obtained.

Construction of the (+/−) Substrate

Linearization. To produce the (+/−) heteroduplex substrate, 100 μg pGem_IDLinker39 is digested with *Bsa*I (New England Biolabs) and 100 μg pGem_IDLinker40 with *Ban*II (1 U enzyme/μg DNA in 500 μl volume) (New England Biolabs). To produce the (+/−) homoduplex substrate, 100 μg pGem_IDLinker39 is digested with *Bsa*I and 100 μg pGem_IDLinker39 with *Ban*II. Completeness of the digest is checked on a 1% agarose gel (Fig. 2, lane 1) and, if complete, the DNA is ethanol precipitated and then dissolved in 500 μl 10 m*M* Tris–HCl, pH 8.0.

Annealing. The annealing is performed in 3 ml total volume containing 10 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 7.6, 100 μg pGem_IDLinker40 (*Ban*II), and 100 μg pGem_IDLinker39 (*Bsa*I) to produce

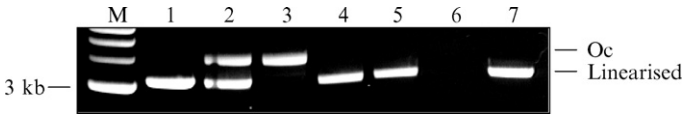

FIG. 2. Production of the (+/−) substrate. Aliquots of the various steps in the preparation of this substrate were separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualized on a UV transilluminator. M, 1-kb DNA ladder; lane 1, 1 μl before annealing; lane 2, 2 μl after annealing; lane 3, 40 ng after DNase V digest and precipitation; lane 4, 40 ng after *Nde*I digest; lane 5, 40 ng after Klenow fill in and Sephadex purification; lane 6, double volume of DNA sup; and lane 7, 40 ng linearized pGemIDLinker40. The mobilities of open circular (oc) and linearized DNA molecules are shown on the right.

the (+/−) heteroduplex substrate or 100 μg pGem_IDLinker39 (*Ban*II) and 100 μg pGem_IDLinker39 (*Bsa*I) to produce the (+/−) homoduplex substrate. Ninety microliters of freshly prepared 10 *N* NaOH (200 mg in 500 μl H_2O) is added, and the solution is mixed gently and left at room temperature for 5 min. The following solutions are then added sequentially: 300 μl 2.9 *M* acetic acid, 135 μl 3 *M* KCl, 372 μl 1 *M* potassium phosphate, pH 7.4. The solution is incubated for 30 min at 65°, then for 3 h at 37°, and finally left to stand at room temperature while 2 μl of the reaction mix are loaded onto a 1% agarose gel to check for annealing efficiency (Fig. 2, lane 2). The nicked (+/−) substrates run slightly slower than the linearized DNA.

Purification. The annealing mixture is dialyzed for 2 × 1 h against 1 liter of 10 m*M* Tris–HCl, pH 8, 0.1 m*M* EDTA. The linear side product of the reaction is removed by the addition of DNase V and incubation at 37° overnight in 33 m*M* Tris–acetate (stock solution: 1 *M*, pH 7.8), 66 m*M* potassium acetate (stock solution 3 *M*, pH 7.7), 10 m*M* magnesium acetate (stock solution 1 *M*), 1 m*M* ATP, 0.5 m*M* dithiothreitol (DTT), and 50 U Plasmid-Safe ATP-Dependent DNase (Epicentre). The efficiency of the digest is checked on a 1% agarose gel (Fig. 2, lane 3). If the faster migrating band of linearized DNA has disappeared completely, the DNase V is heat inactivated by incubation at 70° for 30 min. The formerly clear solution now becomes turbid and has to be filtered prior to DNA precipitation. If the filtration step is omitted, the DNA will be only partially soluble after the precipitation. The filtration is performed with PD-10 desalting columns (Amersham Biosciences), which contain Sephadex G-25 (two columns/reaction). The eluate is precipitated with 0.2 *M* NaCl and 2× total volume of ice-cold ethanol, left at −20° for at least 90 min, centrifuged at 20,000*g* for 30 min at 4°, washed with 6 ml 70% ethanol, and centrifuged again at 20,000*g* for 10 min at 4°. The pellets, which may not be visible, are dried at room temperature and then dissolved in about 250 μl 10 m*M* Tris–HCl, pH

8.0. The concentration and quality of the DNA are determined by analytical agarose gels and/or on a Nanodrop. The yield is between 25 and 40 μg (25–40%). If the DNA is to be used in mismatch repair assays, it is purified further by gel extraction.

Biotinylation. If the DNA is to be used as an affinity matrix, it has to be labeled with biotin, such that it will bind onto magnetic, streptavidin-coated beads. To this end, the DNA is first cut with *Nde*I (4 U/μg DNA; New England Biolabs), which creates the required TA-5′ overhangs. The digest is checked on an agarose gel (Fig. 2, lane 4) and, if complete, the enzyme is heat inactivated by incubation at 65° for 30 min. The ends are filled in with Klenow polymerase under the following conditions: 10 μg (+/−) substrate (*Nde*I), 10 U Klenow polymerase (Roche), 33 μM biotin-16–2′-deoxyuridine-5′-triphosphate (Biotin-16-dUTP; Roche), 33 μM of each dATP, dGTP, and dCTP. The reaction is allowed to proceed at 25° for 30 min and is then stopped by the addition of EDTA to a final concentration of 10 mM and heating to 75° for 20 min. The biotinylated substrate is then purified from the excess of free biotin-16-dUTP on a Sephadex G-25 or G-50 filtration column (self-packed). The flow through will contain the purified, biotinylated and nicked (+/−) substrate (Fig. 2, lane 5).

Coating of Magnetic Beads with the (+/−) Substrate DNA. To obtain complete binding of the biotinylated DNA to Dynabeads M-280 streptavidin (Dynal), 30 μl of the bead suspension is used per microgram of (+/−) substrate. The beads are agitated and the desired amount is taken out and aliquoted into 1.5-ml Eppendorf tubes. These are put into a magnetic particle concentrator (MPC; Dynal MPC-E-1) and allowed to attach for about 2 min. The storage buffer is removed. An equal volume of wash buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 M NaCl, 0.003% [v/v] NP-40) is added and the beads are resuspended by flicking the side of the tube. They are then allowed to attach to the wall in the MPC for about 2 min. This procedure is repeated twice. The beads are then washed once with B&W buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 M NaCl, 0.003% [v/v] NP40).

The DNA is attached to the beads as follows: the desired amount of DNA and an equal volume of B&W buffer are added to the beads and the mixture is incubated at 43° for 1 h. The supernatant (DNA supernatant) is then removed from the beads using the MPC. To ensure complete binding of the DNA, the DNA supernatant should be analyzed on an agarose gel (Fig. 2, lane 6). The beads are then washed twice with wash buffer and once with B&W buffer by resuspension and immobilization in the MPC at each step and are now ready to be used for DNA affinity purification.

Construction of 3′ and 5′ Substrates

By definition, the 5′ substrate contains a single-strand nick upstream from the mismatch. On this substrate, the mismatch is removed by a 5′→3′ exonuclease, which starts at the nick. In contrast, the 3′ substrate, which contains the nick downstream from the mispair, has to be processed first by a 3′→5′ exonuclease. The key difference between these substrates is that the mismatch in the former can, in principle, be ''repaired'' by nick translation even in MMR-deficient extracts, whereas the latter cannot.

Production of Bacteriophage M13K07. M13K07 is a helper phage. When host bacteria harbor both phagemid and M13KO7, the gene II product of the phage interacts with the f1 intergenic region present in the phagemid and initiates rolling circle replication to generate copies of one strand of the phagemid DNA (ssDNA). The permissive *Escherichia coli* strains must possess pili to accept the phage. One such suitable strain is *XL1 blue*, which contains a tetracycline selectable F' factor. (When working with phage, caution must be used not to contaminate the environment: sterile tips should be employed, used tips and pipettes should be discarded in 80% ethanol, and flasks and centrifuge bottles should first be washed out with 10 *N* NaOH and then with 80% ethanol).

PRODUCTION OF RECEPTIVE (MALE) XL1 BLUE CELLS. *XL1 blue* cells from a glycerol stock are streaked out on a 2× YT/Tet (5 μg/ml) plate and incubated overnight at 37°. (To produce 1 liter 2× YT medium, 16 g tryptone, 10 g yeast extract, and 5 g NaCl are dissolved in water and the pH is adjusted to pH 7.0 prior to autoclaving. To produce 2× YT plates, 15 g agar are added to 1 liter 2× YT medium prior to autoclaving.) Five single colonies are picked the next day and inoculated into 5 ml of 2× YT/Tet medium (5 μg/ml) each. They are allowed to grow at 37° overnight. (To prepare a new glycerol stock, 10 μl of the overnight culture is added to 5 ml of 2× YT/Tet medium [5 μg/ml] and incubated at 37° for 6 h. Six hundred microliters is then mixed with 400 μl of 50% glycerol and the stocks are stored at −80°.) As the F′ factor can be lost, the clones should be tested for its presence as follows.

A thick line of each overnight culture is streaked on a 2× YT/Tet (5 μg/ml) plate in the morning using a sterile inoculation loop. One microliter of M13K07 phage from a stock with a titer of at least 10^6 is spotted at the beginning of each line (make also one control plate without phage). After 6 h of incubation at 37°, the lines are checked for plaque formation at the site of phage release. Clear transparent spots (plaques) indicate that the respective *XL1 blue* clone is receptive to phage infection. A few cells are lifted with a sterilized Pasteur pipette (without touching the plaques),

streaked out on a fresh 2× YT/Tet (5 μg/ml) plate, and incubated overnight at 37°.

PRODUCTION OF M13K07 PHAGE. Five milliliters of 2× YT/Tet medium (5 μg/ml) are inoculated with a single male *XL1 blue* colony and incubated at 37° for 6–8 h. Five 4 ml aliquots of liquid top agar (autoclaved, melted, aliquoted in snap-cap tubes, and kept a 44°) are prepared. Two hundred microliters of the bacterial culture are added to 100 μl of five different dilutions of M13K07 (or phosphate-buffered saline [PBS] as a blank; reasonable dilutions of a 1.7×10^{13} pfu/ml stock: 10^{-9}–$10^{-12} = 1.7 \times 10^{4}$–$10^{1}$ pfu/ml = 17,000–17 plaques) and incubated for 10 min at 37°. Bacteria (blank) and bacteria/phage mixtures are added to the top agar, mixed briefly, and then poured evenly onto prewarmed 2× YT/Tet (5 μg/ml) plates. The plates are allowed to dry at room temperature for 20 min and then incubated overnight at 37°. After 12–15 h of incubation, the plates should show a confluent opaque bacterial layer with plaques (number should be consistent with the corresponding phage dilutions). One liter of 2× YT/Tet (5 μg/ml)/Kan (30 μg/ml) medium in a 5-liter conical flask is inoculated with one single plaque from the bacterial layer by aspirating a plug containing the selected plaque with a sterile Pasteur pipette and flushing it into the culture medium. The phage culture is incubated overnight at 37° with shaking.

The next day, it is centrifuged at 2700*g* for 30 min at 4°. The pellet is discarded, and the supernatant containing the phage is transferred to new tubes and centrifuged at 10,800*g* for 30 min at 4°. The pellet is discarded and the supernatant is incubated at 70° for 30 min and then left at room temperature for 10 min. The phage solution is centrifuged at 10,800*g* for 10 min at 4° and the supernatant is transferred to a 2-liter conical flask, to which one-fourth of the volume of 20% PEG/2.5 *M* NaCl is added, and the phage particles are precipitated overnight at 4° without shaking.

The next day, the PEG-precipitated phage particles are centrifuged at 10,800*g* for 30 min at 4°. The supernatant is discarded and all traces of it are removed from the neck of the flask with a paper towel. The pellet containing the phages is resuspended in a minimal volume (6–8 ml) PBS, pH 7.4, with shaking for 1 h at room temperature. The suspension is then transferred to Eppendorf tubes and centrifuged at 20,800*g* for 10 min at 4° in a tabletop centrifuge. The supernatant containing the purified phages is then aliquoted (the pellet consisting of residual bacterial cells is discarded) and the aliquots are stored at 4°.

DETERMINATION OF PHAGE TITER. Five milliliters 2× YT/Tet (5 μg/ml) medium are inoculated with a single colony of the tested male strain and allowed to grow at 37°. Six hours later, seven dry 2× YT/Tet (5 μg/ml) plates and 7 × 4 ml liquid sterile top agar aliquots in a 44° water bath are

prepared. Assuming that the new phage stock concentration is 10^{13} pfu/ml, serial dilutions in PBS, pH 7.4, are made and 100 μl of phage dilutions 10^{-7}–10^{-12} (or PBS as a blank) are added to 200 μl of the bacterial culture, mixed briefly, incubated for 10 min at 37°, mixed with 4 ml top agar, and plated onto prewarmed 2× YT/Tet (5 μg/ml) plates. The plates are incubated at 37° for at least 15 h. The plaques on the plates with the higher dilutions are counted, the average value is calculated, and the pfu/ml are calculated (e.g., 50 plaques on the plate with the 10^{-10} dilution corresponds to 500 pfu/ml and thus the stock has a titer of 5×10^{12} pfu/ml).

Production of Single-Stranded DNA. When using plasmids derived from pGemZf(+), the sequence of the ssDNA corresponds to the lower strand shown in the Promega catalog.

Day 1. *XL1 blue* are transformed with 20 pg of the desired plasmid (e.g., pGem_IDLinker40 to produce the ΔT substrate).

Day 2. One hundred millilters of 2× YT/Amp (100 μg/ml)/Tet (5 μg/ml) medium is inoculated with a single transformed colony and incubated overnight at 37° with shaking at 200–250 rpm.

Day 3. The OD_{600} of a 1/10 dilution of the overnight culture is measured. Then, 2× 1 liter of 2× YT/Amp (100 μg/ml)/Tet (5 μg/ml) medium in 5-liter conical flasks is inoculated with the overnight culture such that the OD_{600} at time 0 is 0.07. The cultures are grown at 37° with shaking at 220 rpm, and the OD_{600} is checked every 20 min until OD_{600} reaches 0.2–0.3 (after about 80 min). Then, a superinfection with the helper phage M13K07 is made at a multiplicity of infection (MOI) of 20. Assuming that $OD_{600} = 1$ corresponds to 5×10^8 cells/ml ($= 1.25 \times 10^{11}$/liter), 2.5×10^{12} phage particles (250 μl from a 10^{13} phage stock) have to be added per liter. The bacteria are kept for 10 min at 37° without shaking and are then shaken at 220 rpm for 90 min at 37°. After 90 min, 30 μg/ml kanamycin are added to select for M13K07-infected cells. The bacteria are allowed to grow for a further 3 h. The cultures are cooled on ice for 30 min, and the suspension is transferred to centrifuge bottles and centrifuged at 5000*g* for 30 min at 4°. The supernatant is transferred to fresh centrifuge bottles, heated to 70° for 30 min, equilibrated at room temperature for 15 min, and centrifuged at 10,000*g* for 15 min. This step kills and removes remaining bacteria in the suspension. The supernatant is transferred to a 5-liter conical flask and 1/4 volume (~ 500 ml) of 20% (w/v) PEG8'000/2.5 *M* NaCl is added. The phage particles are precipitated overnight at 4°.

Day 4. The phage suspension is transferred into centrifuge bottles and centrifuged at 10,000*g* for 30 min at 4°. The supernatant is discarded and the remaining liquid is removed with paper towels. The precipitated phage particles form a whitish film on the wall of the centrifuge bottles, which is

hardly visible. These are resuspended in a minimum volume of PBS, pH 7.4 (about 15 ml for 2 liter starting culture), with moderate shaking for 1–2 h at room temperature. The resulting suspension is very dense and brownish and is centrifuged at 24,000*g* for 15 min at 4°. The supernatant containing the phage particles is transferred to fresh centrifuge bottles, and the pellet of residual bacterial cells is discarded. The phage suspension is now opaque yellow.

If *XL1 blue* cells were used, the phage suspension is usually contaminated with chromosomal DNA. To test for this, 2 μl of the phage suspension is loaded onto a 1% agarose gel. To remove the contamination, $MgCl_2$ (15 m*M* final concentration) and 120 U DNase I are added, and the suspension is incubated at 37° for 2 h. The phage capsid proteins will protect the single-stranded phage DNA from the DNase I. Completeness of the reaction is checked by loading 2 μl of the phage suspension before the addition of DNase I and after 2 h of incubation. When the contaminating DNA has been removed, EDTA, pH 8, is added (60 m*M* final concentration) to chelate Mg^{2+} and to block the activity of DNase I. The enzyme is inactivated by heating the solution to 80° for 20 min. The phage particles are resistant to such treatment. When the phage solution has cooled down to 50° or less, proteinase K is added to a final concentration of 100 μg/ml and the solution is incubated at 37° overnight to digest the capsid proteins and release the ssDNA. In the course of this process, the solution should become clear.

Day 5. A 1/10 volume of 5% hexadecyltrimethylammonium bromide (CTAB)/0.5 *M* NaCl (to prepare a 100-ml stock solution, 2.92 g NaCl are dissolved in 80 ml water, 5 g CTAB are added slowly with stirring, and the solution is then heated to 65°; water is added to a final volume of 100 ml) is added to the proteinase K digestion from day 4, kept for 10 min at room temperature, and centrifuged at 10,000*g* for 20 min at room temperature. The pellet is dissolved in 5 ml 1.2 *M* NaCl, 12.5 ml of cold absolute ethanol is added, and the solution is kept at −20° for at least 1 h. It is then centrifuged at 23,000*g* for 30 min at 4°, and the pellet of ssDNA is rinsed with 5 ml 70% ethanol and centrifuged again for 10 min. This second pellet are significantly smaller and whiter than that from the CTAB/NaCl precipitation. It is air dried and dissolved in about 4 ml 10 m*M* Tris–HCl, pH 8.0, 0.1 m*M* EDTA. The DNA concentration is determined by measuring the OD_{260} (the multiplier for ssDNA is 40). The yield is typically about 0.5 mg ssDNA/2 liters of starting culture.

Annealing and Purification of 3′ and 5′ Substrates. For construction of the 3′ substrate, 200 μg of pGem_IDLinker39 (for 3′ heteroduplex) and 200 μg of pGem_IDLinker40 (for 3′ homoduplex) are digested with *Sap*I.

The resulting 3′ heteroduplex will contain a nick 291 bp 3′ from the deletion site; the 3′ homoduplex will contain the nick at the same site.

For construction of the 5′ substrate, 200 μg of pGem_IDLinker39 (for 5′ heteroduplex) and 200 μg of pGem_IDLinker40 (for 5′ homoduplex) are digested with *Ban*II. The resulting 5′ heteroduplex will contain a nick 369 bp 5′ from the deletion site; the 5′ homoduplex will contain the nick at the same site. Completeness of the digests is checked on a 1% agarose gel and, when complete, the DNA is precipitated and then dissolved in 500 μl 10 m*M* Tris–HCl, pH 8.

Annealing is performed in 3 ml of a buffer containing 10 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 7.6, 200 μg linearized plasmid DNA, and 100 μg ssDNA (mole ratio dsDNA:ssDNA = 1:1). Subsequent steps are performed exactly the same as for the production of the (+/−) substrate. The circular, nicked DNA is purified further by electrophoresis on 0.8% agarose gels to remove the contaminating ssDNA, which interferes with the MMR assays and with the restriction digest by *Nde*I.

Construction of G/T and G/C Mismatch Repair Substrates

Experimental Strategy

The vector used is a derivative of the pGEM-13Zf(+) vector (Promega). The original vector contained six *Ple*I restriction sites (isoschizomer of *N.Bst*NBI), which were removed by QuickChange site-directed mutagenesis (Stratagene) and appropriate primers. Furthermore, the linker sequence 5′-GGCCGCGATCTGATCAGATCCAGACG TCTGTCAACGTTGGGA-3′ was cloned between its *Not*I and *Hin*dIII restriction sites. Using site-directed mutagenesis, a single recognition site for the nickase *N.Bst*NBI (New England Biolabs) was inserted, which is situated either 352 bp 3′ from the mismatch if the complementary (top) strand is nicked or 337 bp 5′ from it when the viral (bottom) strand is nicked (Fig. 3).

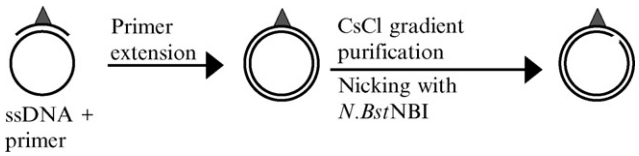

FIG. 3. Schematic representation of the production of mismatch repair substrates by the primer extension method.

To construct the hetero- and homoduplex substrates, the following PAGE-purified primers were used (Microsynth GmbH, Switzerland) in primer extension reactions:

- for G/T: 5′-CCAGACGTCTGTCGACGTTGGGAAGCTTGAG-3′
- for G/C: 5′-CCAGACGTCTGTCAACGTTGGGAAGCTTGAG-3′

In this method, oligonucleotides can be used that carry modified bases, e.g., 6-methylguanine, 8-oxoguanine, thymine dimers, and cisplatin adducts. However, it is highly advisable to check the quality of oligonucleotides containing these modifications by mass spectrometry. For example, ^{6Me}G is incorporated into oligonucleotides as a 6-*O*-methyl-2-*N*-isobutyryl-2′-deoxyriboside. The isobutyryl protecting group does not come off by ammonia treatment (standard deprotection procedure for synthetic oligonucleotides) and has to be removed with (1,8-diazabicyclo [5.4.0] undec. -7-enc (DBU) (a strong base) in anhydrous methanol over the course of several days in the dark. This procedure is often incomplete and the partially protected primers reduce primer extension yields and interfere with the MMR assay.

Phosphorylation of Primers

The primers are phosphorylated in a 100-μl reaction volume containing 4 nmol of the corresponding primer, 1× polynucleotide kinase (PNK) buffer, 5 m*M* DTT, 1 m*M* ATP, 100 μg/ml bovine serum albumin (BSA), and 10 U T4 DNA PNK. The reaction is incubated for 1 h at 37°, followed by 10 min at 70° to inactivate the enzyme. One hundred microliters of 2 *M* $LiClO_4$ and 800 μl acetone are added, and the mixture is placed on ice for 30 min and then centrifuged at 20,800*g* for 20 min at room temperature in a tabletop centrifuge. The pellet is washed with 1 ml 80% acetone and centrifuged at 20,800*g* for 10 min at room temperature. It is dried and dissolved in 100 μl 10 m*M* Tris–HCl, pH 8.0, 0.1 m*M* EDTA.

Primer Extension

Sixty micrograms of the ssDNA (57 pmol) is annealed with a 2.1-fold molar excess (120 pmol) of the appropriate primer in a total volume of 240 μl containing 1× T4 DNA polymerase reaction buffer. The solution is heated for 6 min at 85° in a heat block and then allowed to slowly cool down to room temperature. The extension is done in a 600-μl reaction volume in 1× T4 DNA polymerase reaction buffer containing 100 μg/ml BSA, 1 m*M* ATP, 1 m*M* dNTPs, 2400 U T4 DNA ligase, and 54 U T4 DNA polymerase. The reaction is incubated for 1 h at 37°, followed by incubation at 70° for 20 min to inactivate the enzymes. The efficiency of the reaction is checked on a 1% agarose gel (Fig. 4, lane 3).

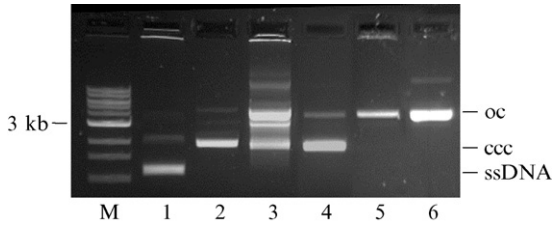

FIG. 4. Production of hetero- and homoduplex MMR substrates by the primer extension method. Aliquots of the various steps in the production of these substrates were separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualized on a UV transilluminator. M, 1-kb DNA ladder; lane 1, 110 ng ssDNA; lane 2, Miniprep DNA; lane 3, 1 μl primer extension G/T; lane 4, 0.5 μl primer extension G/T after cesium chloride gradient; lane 5, nicked Miniprep DNA; lane 6, nicked G/T substrate. The mobilities of open circular (oc), covalently closed circular (ccc), and single-stranded (ss) DNA molecules are shown on the right.

Cesium Chloride Gradient Purification

Twelve grams of CsCl are dissolved in 10.5 ml TE buffer, pH 8.0, and 240 μl ethidium bromide solution (10 mg/ml) are added. The DNA is nicked in the presence of high ethidium bromide concentrations and light and thus the ethidium bromide- and DNA-containing solutions should be kept in the dark. The primer extension reaction is transferred into a 6-ml ultracentrifuge tube (Sorvall S/L, Sleeve Conical), and the tube is filled to the top with the CsCl/ethidium bromide solution; bubbles are removed and the tube is sealed. The counterweight is prepared in the same way; the weight difference has to be $\leq$5 mg. The CsCl gradient centrifugation is then performed with a Sorvall Stepsaver 65V13 rotor at 60,000 rpm for 16–18 h at 4° (with slow deceleration below 20,000 rpm). The centrifuge tube, fastened in a clamp in the darkroom, is pierced at the top with a 25-gauge needle to allow air to enter. An ultraviolet (UV) lamp is switched on and the bright orange band that appears about midway of the tube is sucked out with a 18-gauge needle and a 1-ml syringe by puncturing the tube slightly below the substrate band. The needle is removed and the DNA is ejected into a 2-ml Eppendorf tube containing 700 μl water-saturated *n*-butanol. The solution is mixed by inverting the tube gently, and the upper phase is then removed and the lower phase containing the DNA is transferred to the next Eppendorf tube. This is repeated until the DNA-containing phase is completely colorless (about six times). The DNA is dialyzed for 1 and 3 h against 1 liter of 10 m*M* Tris–HCl, pH 8.0, 0.1 m*M* EDTA. Afterward, the DNA is precipitated in 0.3 *M* NaOAc, pH 5.5, and 2 volumes of ice-cold ethanol, left at −20° for at least 1 h, and centrifuged. After drying, the

pellet is redissolved in 90 μl 10 m*M* Tris–HCl, pH 8.5. The purified substrate is examined on a 1% agarose gel (Fig. 4, lane 4).

Nicking of the Substrate

The purified substrate is nicked with the nicking enzyme *N.Bst*NBI (New England Biolabs) at position 352 in the top (3′) or 337 in the bottom (5′) strand. The nicking reaction is performed in an 80-μl volume containing 45 μl purified substrate, 0.1 mg/ml BSA, 1× *N.Bst*NBI buffer, and 50 U *N.Bst*NBI and incubation is carried out overnight at 16°. One microliter is loaded on a 1% agarose gel to check for completeness of the reaction. If complete, the DNA is purified employing the MinElute reaction cleanup kit (Qiagen). The DNA is eluted in 20 μl 10 m*M* Tris–HCl, pH 8.5, and the volume is adjusted to 80 μl with the same buffer. The concentration and quality of the final substrate are determined with analytical agarose gels (Fig. 4, lane 6) and/or a Nanodrop.

Preparation of Nuclear Extracts

Nuclei are isolated as described (Iaccarino *et al*., 1998) and are resuspended in 500 μl cold extraction buffer/1.5 ml packed nuclei (extraction buffer: 25 m*M* HEPES/KOH, pH 7.5, 10% sucrose, 1 m*M* phenylmethylsulfonyl fluoride [PMSF], 0.5 m*M* DTT, 1 μg/ml leupeptine, 1 tablette/5 ml of protease inhibitor cocktail ''Complete Mini'' [Roche]). The volume of resuspended nuclei is measured, and 0.031 volume of cold 5 *M* NaCl (final concentration of NaCl is 0.155 *M*) is slowly (1 drop/min) added with gentle stirring in the cold room. The mixture is then rotated for 1 h at 4° to allow the proteins to leave the nucleus. After this, nuclei are pelleted by centrifugation at 14,500*g* for 20 min at 2° in a tabletop centrifuge. The supernatant is transferred to a dialysis bag and dialyzed for 2× 1 h against 2 liters cold dialysis buffer (25 m*M* Hepes/KOH, pH 7.5, 50 m*M* KCl, 0.1 m*M* EDTA, 10% sucrose, 1 m*M* PMSF, 2 m*M* DTT, 1 μg/ml leupeptine). The extract is clarified by centrifugation at 20,000*g* for 15 min at 2° in a tabletop centrifuge. The supernatant is aliquoted, snap frozen in liquid nitrogen, and stored at −80°. The concentration of the proteins is determined with a Bradford assay, and the salt concentration is determined using a conductivity meter.

In Vitro Mismatch Repair Assays

Mismatch Repair Assays with G/T and G/C Substrates

Restriction enzymes and positions of the restriction sites used in the interpretation of MMR efficiency are shown in Fig. 5. The G/T substrate is

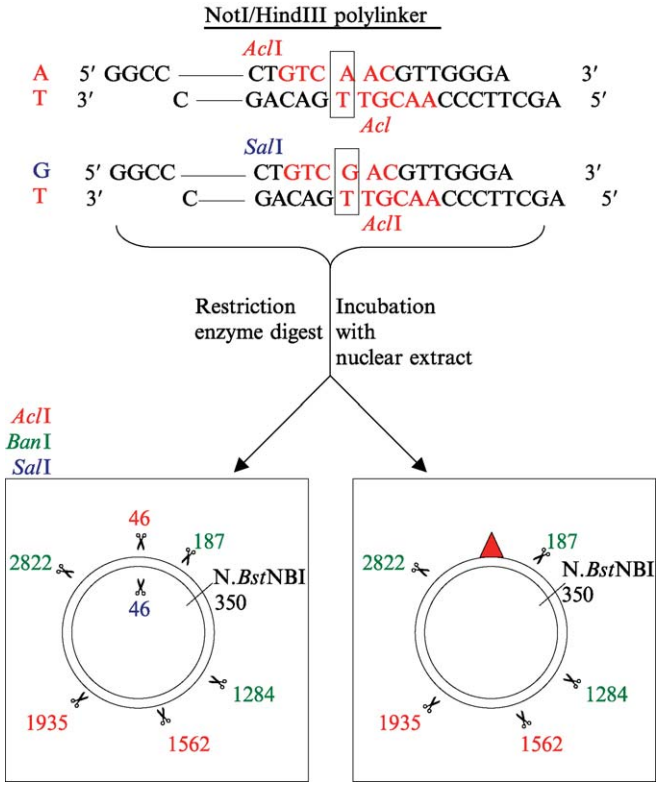

FIG. 5. Schematic representation of the G/T and G/C substrates. The relevant restriction sites and their nucleotide positions are shown.

refractory to cleavage with *Acl*I and *Sal*I. If the G/T mismatch is repaired to A/T, an *Acl*I recognition sequence is restored (*Acl*I cuts the plasmid now three times instead of twice). When the G/T is repaired to G/C, a unique *Sal*I recognition site is restored.

In vitro mismatch repair assays are carried out in 20-μl volumes containing 20 m*M* Tris–HCl, pH 7.6, 110 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* glutathione, 50 μg/ml BSA, 0.1 m*M* each dNTP, 1.5 m*M* ATP, 5 ng/μl substrate (total 100 ng $\cong$ 48 fmol), and 3.75 μg/μl nuclear extract (total 75 μg). Generally, a 10$\times$ MMR buffer containing 200 m*M* Tris–HCl, pH 7.6, 400 m*M* KCl, 50 m*M* $MgCl_2$, 10 m*M* glutathione, 500 μg/ml BSA, and 1 m*M* each dNTP (if the assay is to be performed with [α-^{32}P]dATP, dATP has to be omitted) is prepared, and 20-μl aliquots are made and stored at -20°. These aliquots are only thawed once and kept for less than a month.

In the final reaction, the concentration of KCl is adjusted to 110 m*M* with 1 *M* KCl. We observed that the reaction works well even if only 1.7 pmol [α-^{32}P]dATP together with dGTP, dTTP, and dCTP are included in the reaction. It should thus be noted that, despite the dialysis, the nuclear extracts still contain a residual pool of dNTPs, which permits DNA synthesis.

For the radioactive MMR assay, 10× MMR buffer (-dATP), 1 *M* KCl, DNA substrate, 15 m*M* ATP, 1.7 pmol [α-^{32}P]dATP 10 μCi, and the nuclear extract are mixed to reach the aforementioned concentrations. For the cold MMR assay, 10× MMR buffer containing all four dNTPs is used and the [α-^{32}P]dATP is omitted. The MMR reaction is incubated for 45 min at 37° and is then stopped by adding 30 μl freshly prepared stop solution (1.12% SDS, 41.67 m*M* EDTA, 83.33 μg/ml proteinase K) and incubated again for 30 min at 37°. The DNA is purified employing the MinElute reaction cleanup kit (Qiagen). The eluted DNA is digested with the appropriate restriction enzymes in a 50-μl volume for 3 h at 37°.

The RNA in the samples is removed by adding 2.5 μg RNase A (taking care to eliminate any contaminating DNase activity of the RNase A preparations before use) and incubation for 15 min at 37°. Then, 0.2% SDS and 3.6 μg proteinase K are added and the mixture is incubated for 15 min at 37°. The DNA is precipitated with 0.3 *M* NaOAc, pH 5.5, and 2.5× volumes cold 100% ethanol. The pellet is dissolved in 10.5 μl 10 m*M* Tris–HCl, pH 8.5, and 6× bromphenol blue loading buffer (15%[w/v] Ficoll 400, 0.25% [w/v] bromphenol blue) is added to a final concentration of 1.5-fold. The DNA is loaded onto a 1% agarose gel and run for 45 min at 90 V. The DNA can be visualized in a UV transilluminator (Fig. 6, left); in case of radiolabeled assays, the gel is vacuum dried at 80° for 75–90 min and autoradiographed using a PhosphoScreen (Molecular Dynamics) (Fig. 6, right).

Mismatch Repair Assay with Insertion/Deletion Substrates

In vitro MMR assays are carried out basically as described earlier except for monitoring with the restriction enzymes. The efficiency of repair of the (+/−) substrates is checked by subjecting the DNA to a restriction digest with *Bsa*I (New England Biolabs), which linearizes the DNA substrates, and with the sensor enzymes *Avr*II and *Bgl*II (both New England Biolabs), which cut only when deletion (in the case of *Avr*II) or insertion (in the case of *Bgl*II) occurs. The two fragments (1833 and 1361 bp) indicative of repair are visualized on 0.8% agarose gels.

The efficiency of repair of 3′ and 5′ substrates is visualized by subjecting the DNA to restriction digest with the linearizing enzyme *Bsa*I and the

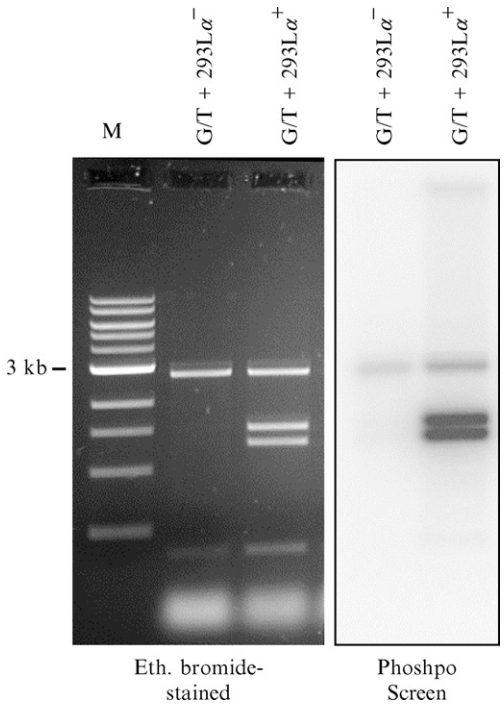

FIG. 6. Repair of the G/T substrate *in vitro*. The substrate containing a G/T mispair in the *AclI*/*Sal*I site of the phagemid substrate was incubated with MMR-proficient or -deficient nuclear extracts in the presence of [α-^{32}P]dATP and digested with the appropriate restriction enzymes to test for efficiency of the repair reaction and incorporation of radioactivity into the different fragments. After the MMR assay, the restriction fragments were separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualized on a UV transilluminator (left). The dried gel was subsequently exposed to a PhosphoScreen (right).

single sensor enzyme *Bgl*II. As the 3′ and 5′ substrates contain the extrahelical ΔT on the continuous strand, insertion occurs almost exclusively.

DNA Affinity Purification

DNA-coated beads are washed twice with buffer W110 (20 m*M* Tris–HCl, pH 7.6, 110 m*M* KCl, 5 m*M* $MgCl_2$) and once with equilibration buffer (1× MMR buffer adjusted to 110 m*M* KCl) while the MPC is standing on ice. The conditions to allow binding of the proteins to DNA are 1× MMR buffer, total of 110 m*M* KCl, 1.5 m*M* ATP, and 360 μg of nuclear extract per microgram of DNA. First, the components without the nuclear extract are added to the beads and incubated for 5 min at the

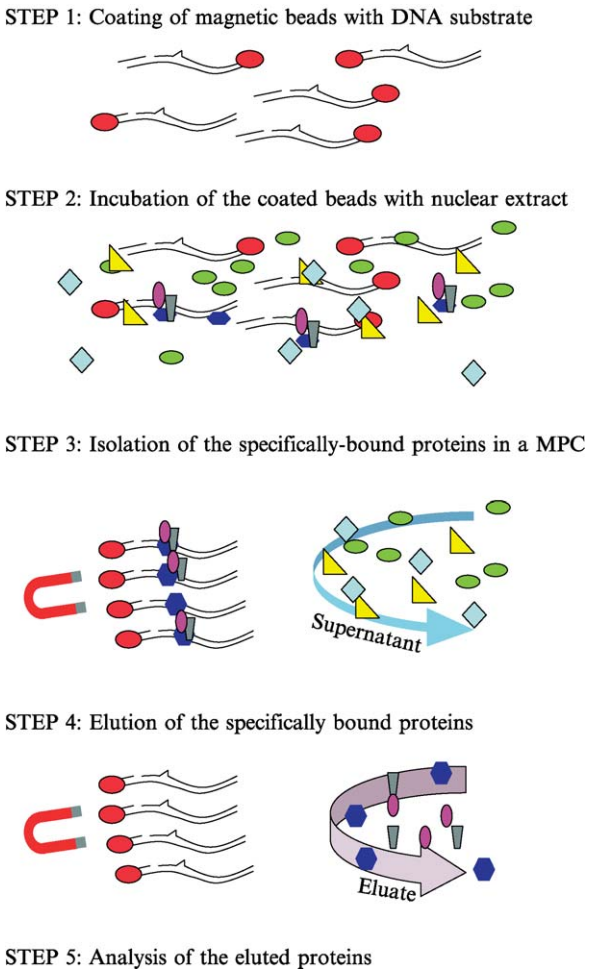

FIG. 7. Scheme of the DNA affinity purification of the "mismatch repairosome."

desired temperature. The nuclear extract is then added and incubated with the DNA for the desired time at the desired temperature. After this, the tubes are put into the cold MPC and the beads are allowed to attach. The supernatant is withdrawn and the beads are washed twice with equilibration buffer and once with buffer W110, buffer W200 (20 m*M* Tris–HCl, pH 7.6, 200 m*M* KCl, 5 m*M* $MgCl_2$), and DNase buffer (20 m*M* Tris–HCl, pH 7.6, 110 m*M* KCl, 10 m*M* $MgCl_2$). Proteins are eluted by cleavage of the

DNA with DNase I by adding 0.9 U DNase I (Roche) in 15 μl DNase buffer per microgram of DNA and incubation at 37°. The DNase I solution is usually added in two portions with incubation at 37° for 10 min after each addition. The eluate is then put into the MPC once more, as some beads are usually cotransferred. The supernatant of this step constitutes the eluate, which is either subjected directly to PAGE or precipitated with 20% TCA (Fig. 7).

Conclusions

The eukaryotic MMR is a complex interplay of numerous polypeptides. Given that MMR malfunction is linked to human cancers, both inherited and sporadic (Jiricny and Marra, 2003; Truninger *et al*., 2005), detailed understanding of the molecular transactions that take place during the recognition and metabolism of the various types of MMR substrates may help identify new genetic loci that predispose to malignancy. Moreover, identification of new interaction partners of the individual MMR factors may lead to the discovery of new metabolic pathways that involve these versatile polypeptides. Thus, in addition to mismatch correction, MMR proteins and their homologs are involved in genetic recombination, both meiotic and mitotic (Harfe and Jinks-Robertson, 2000; Lipkin *et al*., 2002; Snowden *et al*., 2004), and in somatic hypermutation and class switch recombination of immunoglobulin loci (Neuberger *et al*., 2005). The role of MMR proteins in DNA damage signaling is another process that is highly relevant to human health, as MMR malfunction can result in substantial resistance to cancer chemotherapy (Stojic *et al*., 2004). The methods described in this chapter permit the study of the interactions of the MMR system with DNA modifications such as those generated in genomic DNA by chemotherapeutics.

Acknowledgments

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[19] Analysis of DNA Mismatch Repair in Cellular Response to DNA Damage

By LIYA GU and GUO-MIN LI

Abstract

Significant advances have been made in identifying and characterizing the roles of DNA mismatch repair (MMR) proteins in cellular response to DNA damage. Insights into this process have been obtained by performing interactions of mismatch recognition proteins (e.g., MutSα) with DNA adduct-containing duplexes and by analyzing cellular responses (including

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Research Article

Expression of the MutL Homologue hMLH3 in Human Cells and its Role in DNA Mismatch Repair

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Abstract

The human mismatch repair (MMR) proteins hMLH1 and hPMS2 function in MMR as a heterodimer. Cells lacking either protein have a strong mutator phenotype and display microsatellite instability, yet mutations in the *hMLH1* gene account for ~50% of hereditary nonpolyposis colon cancer families, whereas *hPMS2* mutations are substantially less frequent and less penetrant. Similarly, in the mouse model, *Mlh1*$^{-/-}$ animals are highly cancer prone and present with gastrointestinal tumors at an early age, whereas *Pms2*$^{-/-}$ mice succumb to cancer much later in life and do not present with gastrointestinal tumors. This evidence suggested that MLH1 might functionally interact with another MutL homologue, which compensates, at least in part, for a deficiency in PMS2. Sterility of *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$, and *Mlh3*$^{-/-}$ mice implicated the Mlh1/Pms2 and Mlh1/Mlh3 heterodimers in meiotic recombination. We now show that the hMLH1/hMLH3 heterodimer, hMutLγ, can also assist in the repair of base-base mismatches and single extrahelical nucleotides *in vitro*. Analysis of hMLH3 expression in colon cancer cell lines indicated that the protein levels vary substantially and independently of hMLH1. If hMLH3 participates in MMR *in vivo*, its partial redundancy with hPMS2, coupled with the fluctuating expression levels of hMLH3, may help explain the low penetrance of *hPMS2* mutations in hereditary nonpolyposis colon cancer families. (Cancer Res 2005; 65(23): 10759-66)

Introduction

Mismatch repair (MMR) proteins are a highly conserved group of polypeptides that play key roles in the correction of mispairs arising during DNA replication. They also prevent recombination between nonidentical sequences and participate in the signaling of certain types of DNA damage. The importance of MMR proteins in the maintenance of genomic integrity is underscored by the finding that germ line mutations in *MMR* genes predispose to hereditary nonpolyposis colon cancer, a common familial cancer predisposition syndrome (reviewed in refs. 1, 2). The principal MMR players in human cells are homologues of the bacterial MutS and MutL proteins. hMutSα, a heterodimer of the MutS homologues hMSH2 and hMSH6, binds base-base mismatches and small insertion/deletion loops, whereas hMutSβ (a heterodimer of hMSH2 and hMSH3) binds only insertion/deletion loops. This *in vitro* evidence could be corroborated by analysis of the phenotypes of MMR-deficient cells: Those lacking hMSH2 are fully MMR deficient and display a mutator phenotype and microsatellite instability that is consistent with the loss of repair of both base-base mismatches and insertion/deletion loops. Cells lacking hMSH6 retain a strong mutator phenotype but their microsatellite instability is limited to mononucleotide repeats due to the functional redundancy with hMutSβ in insertion/deletion loop repair. This situation is mirrored in hereditary nonpolyposis colon cancer families, where the penetrance of *hMSH2* mutations is substantially higher than that of alterations in the *hMSH6* locus (reviewed in ref. 2).

Whereas it is generally accepted that hMutSα and hMutSβ are the mismatch recognition factors that initiate MMR (reviewed in ref. 3), the function of the MutL homologues remains speculative. The human genome contains numerous genes that have significant sequence homology to *mutL* and to yeast MutL homologue and postmeiotic segregation genes; however, to date, only hMutLα, a heterodimer of hMLH1 and hPMS2, could be shown to be involved in MMR. Correspondingly, hMLH1- or hPMS2-deficient cells have a strong mutator phenotype and high microsatellite instability (reviewed in ref. 1). In *in vitro* studies, hMutLα could be shown to associate with hMutSα on a mismatch-containing substrate (4) and was suggested to act as a "molecular matchmaker" between these protein complexes and the downstream effectors of repair (reviewed in ref. 3). hMutLβ, a heterodimer of hMLH1 and hPMS1, has been biochemically characterized but could not be shown to participate in MMR *in vitro* (5). This finding was substantiated by *in vivo* evidence: Mice carrying a disruption in the *Pms1* gene display neither microsatellite instability nor cancer predisposition (6). hMLH3 was identified through its interaction with hMLH1 on Far Western blots (7); however, this heterodimer, hMutLγ, has not been biochemically characterized and its role in mammalian MMR has not been established. *MLH3* was first identified in *Saccharomyces cerevisiae* and its gene product, scMlh3p, was shown to bind scMlh1p (8, 9) and to be involved in meiotic recombination (reviewed in refs. 10, 11). As *mlh3* mutants display a mutator phenotype similar to that of *msh3*-deficient strains (8, 12), it was suggested that the two polypeptides are involved in the repair of a subset of insertion/deletion loops. hMLH3 seems to be involved in meiotic recombination (13, 14) and the same is true for the murine Mlh3 (14). As both Mlh1- and Mlh3-deficient mice are sterile (reviewed in refs. 10, 11), it was suggested that the two polypeptides function together. However, unlike *Mlh1*$^{-/-}$ animals (6), *Mlh3*$^{-/-}$ mice did not succumb to cancer in the first 9 months of life (15). The roles of the various MMR factors and the phenotypes of mice with defects in *MMR* genes are listed in Table 1.

The involvement of MutL homologue malfunctions in human cancer is not as clear cut as in the case of the MutS homologues. Mutations in *hMLH1* predominate in hereditary nonpolyposis

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Table 1. Overview of mammalian MutS and MutL homologues and their roles in MMR

Heterodimer	Components	MMR role	Phenotype of knockout mice
hMutSα	hMSH2	Repair of base-base mismatches and small loops	Lymphomas, gastrointestinal, skin, and other tumors
	hMSH6		Lymphomas, gastrointestinal, and other tumors
hMutSβ	hMSH2	Repair of loops	Lymphomas, gastrointestinal, skin, and other tumors
	hMSH3		Gastrointestinal tumors
hMutLα	hMLH1	Repair of all MMR substrates	Lymphomas, gastrointestinal, skin and other tumors; sterility
	hPMS2		Lymphomas, sarcomas; male sterility
hMutLβ	hMLH1	?	Lymphomas, gastrointestinal, skin, and other tumors
	hPMS1		No phenotype
hMutLγ	hMLH1	?	Lymphomas, gastrointestinal, skin, and other tumors
	hMLH3		Sterility

colon cancer, accounting for nearly 50% of all known germ line *MMR* gene mutations (2). Surprisingly, no germ line mutations have been found in *hPMS1* or *hPMS2*, which was unexpected, given the key role of the latter protein in MMR. Recent immunohistochemical analysis of 1,048 unselected colon tumors revealed the lack of hPMS2 in ~1.5%, a proportion similar to that of MSH2-deficient cancers (16). Genetic analysis identified germ line mutations in *hPMS2* in a number of these patients and it is likely that the remainder will also be linked to genetic alterations once the problems associated with sequencing of the *hPMS2* locus are overcome (there are ~20 *hPMS2* pseudogenes on chromosome 7, which interfere with DNA sequencing). However, these patients do not belong to typical hereditary nonpolyposis colon cancer families and the penetrance of these mutations seems to be very low. One possible explanation for this finding is that the defect in hPMS2 is partially compensated for by another MutL homologue, such as hMLH3. Germ line *hMLH3* missense and frameshift mutations have been described in familial colorectal cancer cases but the implication of these alterations in carcinogenesis is ambiguous. In some cases, the mutation in *hMLH3* was identified in families carrying a second *MMR* gene mutation, whereas no mutations in the other *MMR* genes could be identified in other cases (17–19). A similar discrepancy applies also to the microsatellite instability status of the tumors (17, 20). The role of hMLH3 in MMR and of *hMLH3* mutations in cancer thus remains open to question. In an attempt to provide answers to these questions, we examined the role of hMLH3 in MMR *in vitro*.

Materials and Methods

cDNA Vectors

pFastBac1-His$_6$-hMLH3. The cDNA of *hMLH3* (Swiss-Prot entry Q9UHC1) was used as template for a PCR reaction where $(His)_6$ tag was added at the NH_2 terminus of *hMLH3* using the primers hMLH3fo1 (5′-CGCGGATCCACCATGTCGTACTACCATCACCATCACCATCACG-ATTACGATATCCCAACGACCGAAAACCTGTATTTTCAGGGCATCAAGTG-CTTGTCAGTTGAAG-3′) and hMLH3re1 (5′-ATTTGCCTACTGGTGG-GACC-3′). The fragment was then cleaved with *Bam*H1 and *Pfl*M1 and cloned between the corresponding sites in pFastBac1 (Invitrogen, San Diego, CA).

***pTXB1-hMLH3* (amino acids 961-1,453).** The COOH-terminal part of *hMLH3* cDNA coding for amino acids 961 to 1,453 was amplified by PCR from pFastBac1-His$_6$-hMLH3 using the primers fMLH3-Ct (5′-GG-GAATTCCATATGGAGAACTGTGTGATATCAGAAACTC-3′) and rMLH3-Ct (5′-AAGGCCGCTCTTCCGCACATTGGTGGCTCACAGGGAGGCATG-3′). The PCR product was subcloned between the *Nde*I/*Sap*I sites of pTXB1 (New England Biolabs, Beverly, MA).

Expression of hMutLγ

The Bac-to-Bac baculovirus expression system (Life Technologies, Gaithersburg, MD) was used according to the instructions of the manufacturer. *Spodoptera frugiperda* Sf9 cells (2×10^8; Life Technologies) were infected with either a single recombinant baculovirus or with a combination of two viruses at a multiplicity of infection of 10. Cells were harvested 72 hours after infection and total extracts were prepared as described (21). Partial purification of hMutLγ from Sf9 extracts was done using Ni-NTA agarose (Qiagen, Hilden, Germany), and the QIAexpressionist system was used according to the instructions of the manufacturer using 5 mL of 50% Ni-NTA slurry per 100 mg of protein extract.

hMutLγ was expressed also in bacteria using a bicistronic vector *pET11b-His$_6$-hMLH3/MLH1* (cloning information on request) in the BL21 strain of *Escherichia coli*. After induction of expression at 37°C for 4 hours with 0.4 mmol/L isopropyl-β-D-thiogalactopyranoside, the heterodimer was expressed but was insoluble. Nevertheless, the protein could be used to quantify the relative abundance of hMLH3 in HeLa cells.

hMLH3 Antibody Production and Purification

The COOH-terminal polypeptide of hMLH3 (amino acids 961-1,453) was expressed using the Impact-CN-System (New England Biolabs) in BL21 *E. coli* transformed with *pTXB1-hMLH3* (amino acids 961-1,453). The peptide was purified using fast protein liquid chromatography on a MiniQ 4.6/50 PE column (Amersham Pharmacia, Uppsala, Sweden) and used to immunize rabbits at Eurogentec (Seraing, Belgium). The rabbit polyclonal antibody was then affinity-purified using the COOH-terminal polypeptide immobilized on a nitrocellulose membrane. In brief, 100 μg of the purified polypeptide were blotted onto a nitrocellulose membrane by standard electrophoretic transfer, visualized by Ponceau S staining, and the corresponding band was cut out. The membrane was blocked with 5% nonfat dry milk in TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20] for 60 minutes, incubated with 700 μL of the polyclonal antibody for 4 hours at 4°C, and washed thrice with TBST for 15 minutes. The membrane was then cut into small pieces (1×0.5 cm) and the antibody was eluted from the membrane by incubation for 20 minutes at room temperature in 0.1 mol/L glycine (pH 2.5). The supernatant was collected and the pH was neutralized by an equal volume of 1 mol/L Tris-HCl (pH 8.0). The purified antibody was stored at −20°C in 50% glycerol.

It was used to perform all the experiments described in this study except for the immunoprecipitation of hMLH3 from human cell extracts.

Human Cell Lines and Preparation of Cell Extracts

All the colon cancer cell lines, HEK293, and HeLa cell lines used in this study were obtained from the cell line repository of Cancer Network Zurich. The hPMS2-deficient cell lines HeLa clone 12 (22) and Hec-1A (23) were kindly provided by Dr. Margherita Bignami (ISS, Rome, Italy). The cell line HEK293T was derived from HEK293 by immortalization with adenovirus 5 DNA and transfection with SV40 large T antigen (24). The *hMLH1* gene in this cell line is epigenetically silenced by promoter hypermethylation (25). The 293T Lα cell line was developed in our laboratory (26). In these cells, the *hMLH1* c-DNA was stably integrated under the control of the tetracycline response promoter

using the Tet-Off system (Clontech, Palo Alto, CA). In the absence of doxycycline, these cells express hMLH1 and are MMR proficient. All the cell lines were cultured at 37°C in a 5% CO_2–humidified atmosphere and maintained in the appropriate media. Whole cell extracts from these cell lines were prepared as described (26) without modifications. The origin and MMR status of the cell lines used in this study is listed in Table 2.

Western Blot Analysis

Western blots were done as previously described (26) using the following primary antibodies: our rabbit polyclonal anti-hMLH3 (1:400), anti-hMLH1 and anti-hPMS2 from BD PharMingen (San Diego, CA) (1:4,000 and 1:1,000, respectively), and anti-β-tubulin (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Coimmunoprecipitation Analysis of hMLH1 and hMLH3

HeLa whole cell extract (1 mg) was incubated in a total volume of 500 μL in NP40 Lysis Buffer [50 mmol/L Tris-HCl (pH 8.0), 125 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1× complete protease inhibitory cocktail (Roche Molecular Biochemicals, Basel, Switzerland)] for 3 hours at 4°C with the anti-hMLH1 (6 μg; BD PharMingen) or anti-hMLH3 (10 μg; Santa Cruz Biotechnology) antibodies. The immunoprecipitates were captured by incubation for 30 minutes at 4°C with 50 μL of 50% slurry of Protein A/G PLUS agarose (Santa Cruz Biotechnology). The agarose beads were then washed thrice with cold NP40 Lysis Buffer and the proteins were eluted with SDS sample buffer and subjected to Western blot analysis. Control experiments were done either in the absence of antibody or in the presence of 25 units of Benzonase (Merck, Whitehouse Station, NJ).

Analysis of the *hMLH3* Promoter and Treatment of Cells with 5-Aza-2′-deoxycytidine

The *hMLH3* 5′ flanking region was analyzed for CpG content with the CpG plot software of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/) and its methylation status was evaluated with methylation-specific PCR as described previously (16). Primer sequences for the unmethylated reactions were 5′-GTTGTGTGTAGTTTTTGGAGTTG-3′ (sense) and 5′-CTCCCAACACCTAAAACTAACA-3′ (antisense), which amplified a 229 bp product. The methylation-specific primers were 5′-CGCGTAGTTTTCGGAGTC-3′ (sense) and 5′ CTAAAACTAACGAAACGCACG 3′ (antisense), which amplified a 205 bp product. The PCR conditions are available on request.

To reactivate the expression of *hMLH1* and *hMLH3*, $2{,}5 \times 10^5$ HEK293T cells were seeded on a 78 cm^2 dish on day 0 and treated with 3 μg/mL of 5-aza-2′-deoxycytidine (Fluka, Buchs, Switzerland) on days 2 and 5. The medium was changed 24 hours after each addition of the drug and the cells were harvested on day 8.

Microarray Experiments

Microarray experiments were done as described previously (27). Gray columns in the graphs represent mRNA levels based on raw signals detected in the corresponding cell lines with the Affymetrix HG-U133A microarray.

Mismatch Repair Assays

The assays were done as described previously (28, 29).

Results

Expression of hMutLγ in Sf9 cells and production of anti-hMLH3 antibody. To produce the recombinant hMLH3 and hMutLγ factors, *S. frugiperda* Sf9 cells were infected with baculoviruses carrying cDNAs encoding *hMLH1* and/or *hMLH3*. Infection of Sf9 cells with the *hMLH3* virus alone yielded the protein in an amount that was hardly detectable by Western blotting. The amount of expressed protein was significantly increased when the cells were coinfected with both *hMLH1* and *hMLH3* vectors (Fig. 1*A*), suggesting that the presence of hMLH1 is necessary for the stabilization of hMLH3 in Sf9 cells. This is reminiscent of hMSH6 and hPMS2, both of which require their heterodimeric partners (hMSH2 and hMLH1, respectively) for stability. However, the amount of the recombinant heterodimer obtained was too low

Table 2. Characteristics of the human cell lines used in this study

Cell lines	Origin	MMR status*	MMR protein defect †	Genetic complementation
293	Embryonic kidney epithelium	+		
293T	Embryonic kidney epithelium	−	**hMLH1**, hPMS2, **hMLH3**	
293T Lα+	Embryonic kidney epithelium	+	**hMLH3**	*hMLH1* cDNA
CaCo2	Colon carcinoma	+		
CO115	Colon carcinoma	−	**hMLH1**, hPMS2	
Colo741	Colon carcinoma	+		
CX-1	Colon carcinoma	+		
GP5D	Colon carcinoma	−	**hMSH2**, hMSH6, hMSH3, **hMLH3**	
HCT116	Colon carcinoma	−	**hMLH1**, hPMS2, hMSH3	
HCT116+Ch.3	Colon carcinoma	+	hMSH3	Chromosome 3
HT29	Colon carcinoma	+		
Hec1A	Endometrial adenocarcinoma	−	**hPMS2, hMSH6**	
Hec1A+Ch.7	Endometrial adenocarcinoma	−	**hMSH6**	Chromosome 7
HeLa	Cervical carcinoma	+		
HeLa clone 12	Cervical carcinoma	−	**hPMS2**	
LS411	Colon carcinoma	−	**hMLH1**, hPMS2	
SW48	Colon carcinoma	−	**hMLH1**, hPMS2	
SW480	Colon carcinoma	+		
SW837	Colon carcinoma	+		

*+, MMR proficient; −, MMR deficient (these cell lines are unable to repair both base-base mismatches and insertion/deletion loops, with the exception of Hec1A+Ch.7, which is able to repair insertion/deletion loops).
†The primary alteration of MMR protein expression is reported in bold. Lack of hMLH1 or hMSH2 lead to proteolytic degradation of hPMS2, or hMSH6 and hMSH3, respectively. The *hMSH3* gene in HCT116 cells is mutated as a consequence of the MMR defect. The hMLH3 alterations are those described in this study; other alterations have been reported elsewhere (see text).

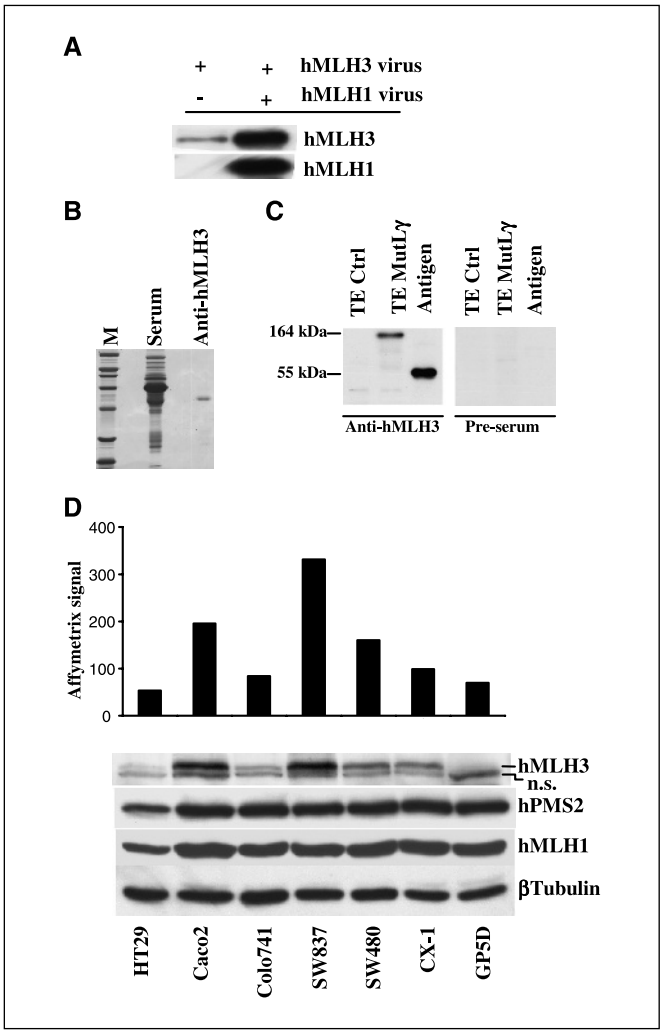

Figure 1. Expression of hMutLγ in Sf9 cells, anti-hMLH3 antibody specificity, and endogenous levels of hMLH3 in colon cancer cell lines. *A,* expression of hMLH3 in Sf9 cells infected either with a baculovirus vector expressing hMLH3 or with a mixture of hMLH1- and hMLH3-expressing viruses. This Western blot shows that hMLH3 is stabilized in this system by hMLH1. *B,* Coomassie blue staining of rabbit polyclonal anti-hMLH3 serum before (*Serum*) and after (*Anti-hMLH3*) affinity purification. *C,* Western blot analysis of whole cell extracts of Sf9 cells uninfected (*TE Ctrl*, 2 μg) or coinfected with the hMLH1/hMLH3 baculoviruses (*TE MutLγ*, 2 μg). The recombinant polypeptide (amino acids 961-1,453) used for the generation of the antibody was loaded in the third lane (1 ng). *Anti-hMLH3,* affinity-purified serum used at 1:400 dilution; *Pre-serum,* preimmune serum used at the same dilution. *D,* microarray analysis of mRNA expression levels (*top*) and Western blot analysis of protein levels (*bottom*) of hMLH3 in a series of colon cancer cell lines (50 μg of whole cell extract per lane); *n.s.,* nonspecific band detected by the anti-hMLH3 antibody in human cell extracts.

to permit extensive purification. The reasons underlying the low levels of expression are unknown at this time, but it is possible that high amounts of the full-length protein may be toxic (7).

Commercially available antibodies could detect the recombinant hMLH3 protein on Western blots but failed to detect the endogenous protein in all the human cell lines used in this study (data not shown). Therefore, we raised our own polyclonal rabbit antiserum, directed against the COOH terminus of hMLH3, which contains the hMLH1-interacting domain. The affinity-purified antibody (Fig. 1*B*) detected a band of the expected size (~160 kDa) in Sf9 lysates infected with the *hMLH1* and *hMLH3* vectors, whereas no signal was visible when we probed lysates of uninfected cells (Fig. 1*C*). The purified antibody was then tested using extracts of various human colon cancer cell lines. The antibody highlighted a double band migrating at the expected size of hMLH3 (Fig. 1*D, bottom*). As the faster migrating band was also observed in Western blots done with the preimmune serum (data not shown), and as the abundance of the slower-migrating band correlated with *hMLH3* mRNA expression levels in the same cell lines (Fig. 1*D, top*), we concluded that the latter is the specific band. As shown in Fig. 1*D*, the levels of hMLH3 fluctuate significantly in the tested cells lines and seem to be independent of the amount of hMLH1 and hPMS2 expressed in the same cells.

Relative abundance of hMLH3 in human cells and its interaction with hMLH1. Given that hMLH3, hPMS2, and hPMS1 interact with the same region of hMLH1 (30), we wanted to ask whether the relative abundance of the three different heterodimers can be correlated with the phenotype of the cells. Therefore, we did semiquantitative Western blots where we compared the intensity of bands due to endogeneous hMLH3 and hPMS2 proteins in HeLa cells with that of bands due to known amounts of the corresponding recombinant proteins (Fig. 2*A*). These experiments revealed that hMLH3 is ~60 times less abundant than hPMS2. Considering that hPMS1 is ~10 times less abundant than hPMS2 in human cells (5), hMLH3 exists in the cells at levels significantly

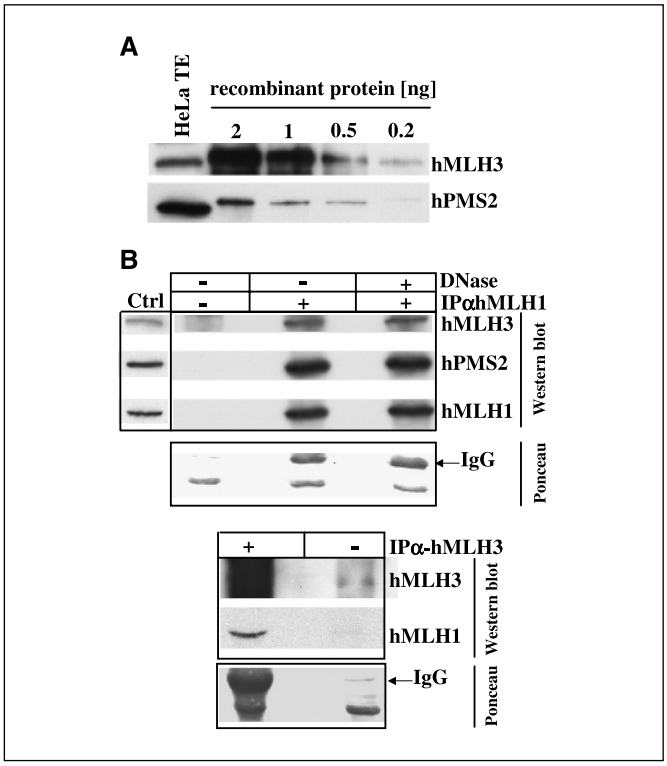

Figure 2. Relative abundance of hMLH3 and its interaction with hMLH1 *in vivo. A,* the recombinant hMLH3 and hPMS2 proteins were loaded onto a denaturing polyacrylamide gel in the indicated amounts and visualized by Western blotting with the respective antibodies. The relative abundance of the two polypeptides was calculated by comparing the intensity of the hMLH3 and hPMS2 bands with those of the endogenous proteins present in 50 μg of HeLa whole cell extract. The blot is representative of two independent experiments, and the intensities of the bands were calculated by densitometry. *B,* coimmunoprecipitation of hMLH3 and hMLH1 in HeLa cells. One milligram of whole cell extract was incubated with or without 6 μg of anti-hMLH1 antibody (*top*) or 10 μg of anti-hMLH3 antibody (*bottom*). *DNase,* reaction done in the presence of 25 units of Benzonase. Ponceau staining for IgG is also shown to show equal loading. *Ctrl,* 50 μg of HeLa whole cell extract.

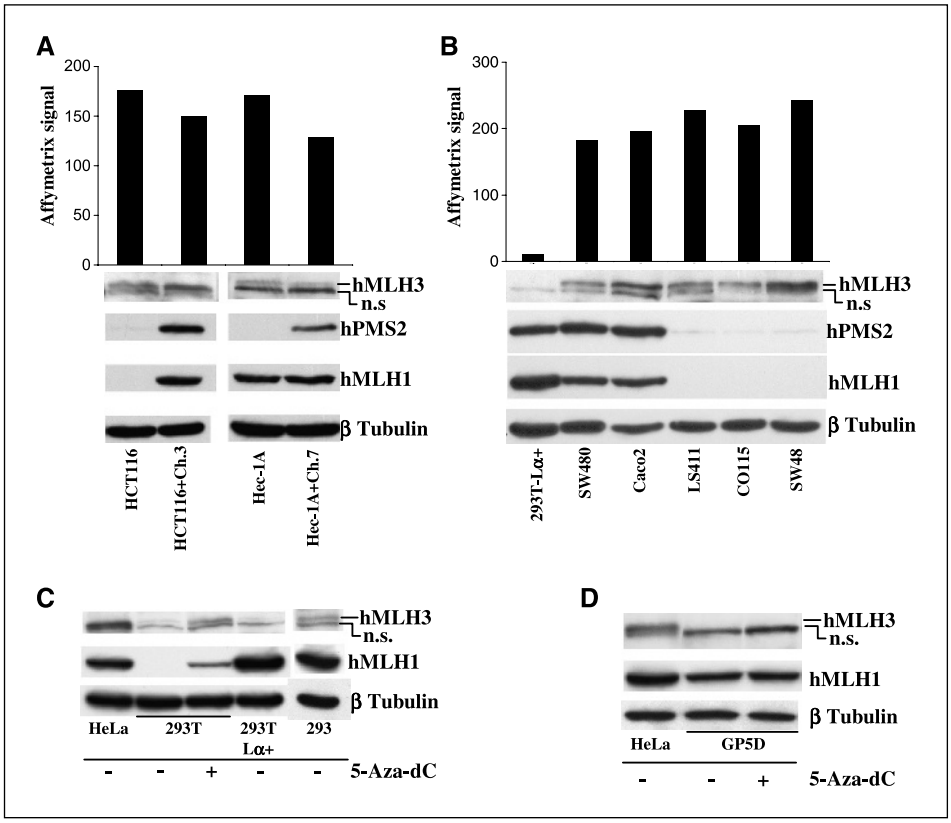

Figure 3. Expression of hMLH3 *in vivo* is independent of hMLH1 and hPMS2 and can be controlled by cytosine methylation. *A,* microarray analysis of *hMLH3* mRNA (*top*) and protein (*bottom*) expression in hMLH1-deficient HCT116 and hPMS2-deficient Hec-1A cells. Correction of the MMR defect by transfer of chromosome 3 or 7, which carry wild-type copies of the *hMLH1* and *hPMS2* genes, respectively, had no effect on hMLH3 expression. *B,* hMLH3 expression is independent of hMLH1/hPMS2 expression in a panel of MMR-proficient and MMR-deficient cell lines. Legend as in (*A*). *C,* the *hMLH3* promoter in 293T cells in silenced by methylation. Expression of *hMLH1* in 293T-Lα cells does not alter hMLH3 levels but demethylation of the promoter by 5-aza-2′-deoxycytidine (*5-Aza-dC*) treatment results in the reappearance of hMLH3, together with hMLH1, the promoter of which is also methylated in these cells. *D,* down-regulation of the *hMLH3* gene in GP5D cells is not mediated by cytosine methylation. The promoter was shown by methylation-specific PCR to be unmethylated and 5-Aza-dC did not reactivate hMLH3 expression in these cells. In the Western blot experiment, 50 μg of total extract were used per lane.

lower than those of the other two hMLH1-interacting partners hPMS2 and hPMS1. In spite of this difference, hMLH3 was found to physically interact with hMLH1 in Far Western experiments (7) and in mammalian two hybrid assays (30). We could confirm this interaction using immunoprecipitation experiments in which the anti-hMLH1 antibody could immunoprecipitate both hMLH3 and hPMS2 from human cell lysates (Fig. 2*B, top*) and the anti-hMLH3 antibody precipitated the endogenous hMLH1 (Fig. 2*B, bottom*). No proteins were detected in control experiments where the precipitating antibody was omitted. The interaction between hMLH3 and hMLH1 was not mediated by bound DNA because treatment with DNase before incubation with the antibodies failed to abolish the interaction between the two proteins (Fig. 2*B, top*).

hMLH1 is not required for the stability of hMLH3 in human cells. hPMS2 and hPMS1 are stabilized by the presence of hMLH1 (1, 31). Considering this characteristic of these two MutL homologues, together with the finding that hMLH1 was required for the stabilization of hMLH3 in baculovirus-infected Sf9 cells (Fig. 1*A*), we expected to observe substantially decreased levels of endogenous hMLH3 in human cell lines lacking hMLH1. Surprisingly, we could detect hMLH3 in hMLH1-deficient HCT116 cells, and the restoration of hMLH1 expression by chromosome 3 transfer resulted in no appreciable increase in hMLH3 level (Fig. 3*A, bottom left*). This shows that the presence of hMLH1 is not required for hMLH3 stability in human cells. The relative amounts of intracellular hMLH3 were also unaffected by hPMS2 levels, as shown by comparison of hMLH3 band intensity in Western blots of extracts of the hPMS2-deficient Hec-1A cells with those of a Hec-1A clone in which the expression of hPMS2 was restored by chromosome 7 transfer (Fig. 3*A, bottom right*). hMLH3 protein levels failed to correlate with hMLH1 and hPMS2 expression also in other colon cancer cell lines, such as SW480 or Caco2, that express both hMLH1 and hPMS2, or LS411, CO115, or SW48 that lack hMutLα (Fig. 3*B*).

Having established that the level of hMLH3 in cells is not dependent on hMLH1 but that it correlates well with *hMLH3* mRNA levels (Fig. 3*B*), we wondered whether the fluctuation of hMLH3 expression in the tested cell lines could be linked with cytosine methylation, which is known to silence several key genes in colon cancer (32). The human embryonic kidney cell line 293T is deficient in both hMLH1 and hMLH3 (Fig. 3*C*) and it could be shown that the CpG islands that constitute the promoters of hMLH1 (25) and other genes (33) are silenced by hypermethylation in these cells. As the *hMLH3* promoter also contains a CpG island, we reasoned that the lack of *hMLH3* expression in this cell line might also be linked to the transcriptional inactivation of its promoter. This prediction was substantiated in two independent experiments. First, treatment of 293T cells with the demethylating agent 5-aza-2′-deoxycytidine partially restored the expression of both hMLH1 and hMLH3 (Fig. 3*C*). In the second experiment, we treated genomic DNA of 293T and the parental 293 cells (which express both hMLH1 and hMLH3; Fig. 3*C*) with sodium bisulfite, which deaminates cytosines to uracils, but leaves 5-methycytosines unchanged. Methylation-specific PCR showed that the promoter of the *hMLH3* gene in 293T cells was indeed methylated (data not shown). As expected, expression of high amounts of hMLH1 in the 293T-derived 293T Lα+ cells resulted in the stabilization of hPMS2 (26) but did not affect hMLH3 levels (Fig. 3*C*). The promoter of the *hMLH3* gene can thus be silenced by cytosine methylation, but this is most likely not the only mechanism that results in the lack of expression of the protein, as 5-aza-2′-deoxycytidine treatment failed to induce the expression of hMLH3 in GP5D cells (Fig. 3*D*).

Role of hMutLγ in *in vitro* mismatch repair. The observation that extracts from 293T-Lα+ cells are MMR proficient (26) despite their lack of hMLH3 suggested that hMutLγ does not play a major role in MMR *in vitro*. However, the possibility that it acts as a backup to hMutLα in the absence of hPMS2 could not be excluded. Therefore, we tested extracts of the human cell line HeLa clone 12, which expresses hMLH1, hPMS1, and hMLH3 but lacks hPMS2. As shown in Fig. 4*A*, these extracts were deficient in the repair of heteroduplex substrates containing either a G/T mismatch or an insertion/deletion loop of one or two nucleotides, but their repair proficiency on all tested substrates could be restored by the addition of recombinant hMutLα. Before concluding that hMutLγ does not participate in MMR, we considered the possibility that the expression level of endogenous hMLH3 in the tested human cell lines might be too low to be detectably active in our *in vitro* assay. Therefore, we decided to test whether *in vitro* MMR activity may be detected in the presence of higher amounts of the heterodimer. These experiments were done with the hMutL α, β, and γ deficient extracts of 293T cells supplemented with whole cell extracts from Sf9 cells expressing comparable amounts of hMutLα or hMutLγ (Fig. 4*B, inset*). As shown previously, extracts of Sf9 cells overexpressing hMutLα could complement the MMR defect in the 293T extracts very efficiently, whereas extracts of uninfected Sf9 cells failed to do so (Fig. 4*B*; ref. 26). Interestingly, when extracts of Sf9 cells expressing hMutLγ were used, we observed an increase in repair activity of ~20%. Similar results were obtained when the hMutLγ was enriched by Ni-agarose chromatography, showing that the observed MMR activity was specific to hMutLγ. We detected similar repair activities on substrates containing a G/T mismatch or a 1-base loop with a nick located either 5′ or 3′ from the mismatch, but no activity was observed on a substrate containing insertion/deletion loops of two or four nucleotides (Fig. 4*B*; data not shown). These experiments show that although physiologic levels of hMutLγ are insufficient to mediate mismatch correction in our *in vitro* MMR assays, the factor can participate, albeit with low efficiency, in the correction of base-base mispairs and one-nucleotide insertion/deletion loops.

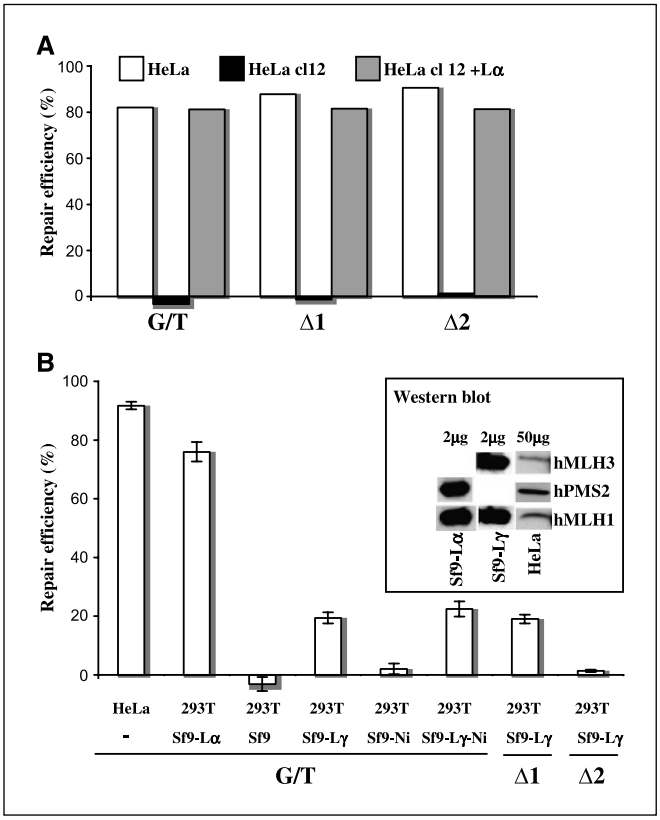

Figure 4. *In vitro* MMR assays. *A,* the MMR defect of cytoplasmic extracts of the hPMS2-deficient cell line HeLa clone 12 can be corrected by the addition of 0.2 μg purified hMutLα. The repair efficiencies were determined on heteroduplex substrates containing a G/T mismatch, or +1 (*Δ1*) or +2 (*Δ2*) insertion/deletion loops. *B,* cytoplasmic extracts of 293T cells, which lack hMLH1, hMLH3, and hPMS2 were supplemented with 2 μg of whole cell extract from Sf9 cells coinfected with hMLH1/hPMS2 (*Sf9-Lα*), hMLH1/hMLH3 (*Sf9-Lγ*), or the latter partially purified on Ni-agarose (*Sf9-Lγ-Ni*). MMR efficiency was tested on heteroduplex substrates containing a G/T mismatch, or +1 (Δ1) or +2 (Δ2) insertion/deletion loops. The amounts of hMutLα and hMutLγ complexes used for complementation were comparable (*inset*). Whole cell extracts from uninfected Sf9 cells (*Sf9*) or Sf9 extract that underwent Ni-agarose (*Sf9-Ni*) chromatography were used as negative controls. *Columns,* result of at least three independent experiments; *bars,* SE. Cytoplasmic extract of the MMR-proficient HeLa cells was used as the positive control.

Discussion

Like its *S. cerevisiae* homologue (8), the mammalian *MLH3* gene (7) could be shown to be involved in meiotic recombination (13–15). However, whereas the *S. cerevisiae* (8) and *Schizosaccharomyces pombe* (12) proteins play a small but distinct role in the repair of a subset of insertion/deletion loops, no similar evidence existed for mammalian MLH3. In this present study, we set out to search for this evidence.

We first wanted to study the expression of hMLH3 and confirm the existence of hMutLγ *in vivo*. Using a newly generated antibody, we showed that hMLH3 is much less abundant than the other two known hMLH1 interactors, hPMS2 and hPMS1. Despite this, we could confirm the physical interaction between hMLH3 and hMLH1 in HeLa cells by immunoprecipitation experiments. Surprisingly, although hMLH1 was required for hMLH3 stability in Sf9 cells (Fig. 1*A*), no such requirement was apparent in human cells where no degradation of hMLH3 occurred in the absence of hMLH1 (Fig. 3*A* and *B*). We also failed to observe any significant competition between hMLH3 and hPMS2 for hMLH1, showing that in human cells hMLH3 might be stabilized by interaction with another, as yet unidentified, protein. This finding is supported by evidence from meiosis in mice, where Mlh3 was seen to bind to pachytene chromosomes before Mlh1 and, after Mlh1 recruitment to these sites, foci containing Mlh3 alone persisted (11, 15). It was, therefore, suggested that Mlh3 could either exist alone or interact with a different partner (11). Immunoprecipitation experiments revealed a direct interaction of *scMlh3p* with *Sgs1* helicase in meiotic *S. cerevisiae* cells (34) and hMLH3 was shown to bind hMSH4 in meiotic human cells (14); however, the identification of the putative hMLH3 partners that might help stabilize it in mitotic cells must await the results of future experiments.

The ultimate objective of this work was to elucidate the role of hMLH3 in human MMR. We first tested extracts of human HeLa clone 12 cells, which lack hPMS2 (22) and thus contain only hMutLβ and hMutLγ. As the former heterodimer is devoid of MMR activity in our *in vitro* MMR assay (31), any observed repair activity could be ascribed to hMutLγ. The extracts were MMR deficient on all tested substrates (Fig. 4*A*), which suggested that the hMutLγ heterodimer does not participate in MMR. However, as hMLH3 is generally much less abundant in human cells than hPMS2, we

wanted to exclude the possibility that the lack of repair activity is linked to insufficient amounts of hMutLγ. Therefore, we tested the MMR activity of extracts of 293T cells, which are deficient in all three MutL homologues, supplemented either with recombinant hMutLα or hMutLγ (Fig. 4*B*). The former factor complemented the MMR defect in the 293T extracts on all tested substrates. When comparable amounts of hMutLγ were used, we observed a small but significant (~20%) repair with both G/T and +1 insertion/deletion loop substrates. This repair activity was not due to an intrinsic repair activity of the Sf9 extracts per se, as extracts from uninfected Sf9 cells were repair deficient in the complementation experiments. As there are no available functional assays to test the activity of hMutLγ, a possibility exists that this heterodimer was isolated in a partially inactive form. However, we consider this possibility unlikely because all the procedures used were identical to those used for the preparation of the Sf9 extract expressing hMutLα, which was fully active. Moreover, immunoprecipitation experiments done with Sf9 extracts expressing hMutLγ showed that hMLH3 was able to bind hMLH1 (data not shown). The sensitivity of the *in vitro* MMR assay remains, however, rather low so that the contribution of hMutLγ to the repair process *in vivo* might be higher. Interestingly, the repair activity of hMutLγ was limited to G/T mismatch and 1-base loops, as we failed to observe any repair activity using +2- and +4-base-loop substrates. The latter result indicates that hMutLγ seems to be involved in the repair of substrates recognized by hMutSα rather than insertion/deletion loops of more than one extrahelical nucleotide recognized by hMutSβ. This is in contrast to the data obtained in *S. cerevisiae* where the role of *scMlh3p* seems to be in the repair of a subset of insertion/deletion loops together with *scMutSβ*. The role of hMLH3 in mammals thus might differ from that in lower eukaryotes.

Our findings, suggesting that hMutLγ may play a backup role in human MMR, are supported by evidence from the mouse model. As noted above, Mlh3 *null* mice were not cancer prone in the first 9 months of life and showed no gross defects in MMR (15). However, a long-term study of these animals, coupled with a highly sensitive analysis of their genomic DNA, provides evidence for the involvement of Mlh3 defects in both MMR and tumorigenesis. $Mlh3^{-/-}$ mice have a shorter life span than the wild-type controls and more than half of the animals develop cancers, including gastrointestinal tumors after the 9-month time span. Importantly, Mlh3 deficiency increased the levels of mutations in long mononucleotide repeats, although to a lesser extent than in $Pms2^{-/-}$ mice (35). Taken together, our results and the mouse model data suggest that the hMutLγ heterodimer functions in the repair of base-base mismatches and small insertion/deletion loops.

Considering the possible involvement of hMLH3 in human MMR, the identification of hMLH3 silencing through promoter hypermethylation is of particular interest. We showed that the hMLH3 promoter is methylated in 293T cells and that the protein is consequently not expressed. In this particular cell line, the methylation could be caused by the presence of the SV40 large T antigen. However, using methylation-specific PCR, we could detect partially methylated hMLH3 promoters in the colon cancer cell line LS411 and in the ovarian cancer cell line A2780/CP70, and fully methylated in the leukemia cell line Jurkat (data not shown), which shows that hMLH3 silencing via promoter hypermethylation can also be unrelated to the presence of SV40 large T antigen.

Although recombinant hMutLγ possessed detectable repair activity in our *in vitro* MMR assays, hPMS2-deficient cells expressing hMLH3 display a strong mutator phenotype (refs. 16, 23; this study). This suggests that hMLH3, most likely in the form of hMutLγ, does not play a major role in MMR *in vivo*. However, the detection of sequence variants of hMLH3 in the germ line of families predisposed to colorectal cancer (17, 20), coupled with our detection of epigenetic silencing of hMLH3 in human cell lines, suggests that this gene may play a role in human cancer, possibly in combination with other risk factors. If hMutLγ does indeed play a backup role for hMutLα *in vivo*, the fluctuating abundance of hMLH3, such as that observed in the tested cell lines (Figs. 1 and 3), might help explain the variable penetrance of *hPMS2* mutations in hereditary nonpolyposis colon cancer families (16).

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7. Conclusions

The mismatch repair system (MMR) is an important determinant in the response to chemotherapeutic agents by mediating the cytotoxic effects of the applied drugs.

O^6-methylguanine (^{m}G), the most cytotoxic lesion induced by S_N1-type methylating agents, is recognized both in ^{m}G/T and ^{m}G/C mispairs by the human MMR factor MutSα. In bandshift experiments, ^{m}G/T and ^{m}G/C were recognized with equally low efficiency, comparable to a homoduplex control oligonucleotide ([1]; our unpublished results). The strongly reduced repair of ^{m}G-containing substrates in our *in vitro* MMR assays mirrored this weak recognition. It has been reported that mismatch recognition by MMR depends on the sequence context and other groups observed similar binding properties of hMutSα to ^{m}G/T and G/T mispairs ([2]; Paul Modrich, personal communication). Although we tested two unrelated sequences in the bandshift experiments, no appreciable differences in the recognition by hMutSα could be observed. Since bandshift experiments are not standardized and therefore performed in a remarkable diversity, this discrepancy may originate from different assay conditions. Another explanation could be that, by mischance, both our sequences are not optimal substrates for an efficient recognition by MMR factors. Probably, this very weak recognition made it impossible to detect a putative minor fraction of molecules undergoing futile repair opposite ^{m}G. This could simply argue for the absence of a futile repair scenario and could be taken as proof of an alternative model. However, given that Paul Modrich's group provided evidence for the former hypothesis *in vitro* by using substrates differing in sequence from ours [3], we believe that the observed discrepancy between the two sets of data is due to different efficiencies of the initial recognition of the lesion. This could also account for the fact that no single-stranded regions were visualized opposite ^{m}G in our system. Furthermore, no concluding statement about which nucleotide is inserted across the lesion could be made. The *in vitro* system used in this study turned out to exert a variety of unpredictable and complex activities on our substrates, making the study of single events extremely difficult. In addition, substrate preparations varied in our hands and showed slightly different

behavior in the MMR assays. The reconstituted systems, on the other hand, run the risk of showing experimental artefacts due to the enormous restriction to a small subset of proteins. Since MMR has been shown to work in *Xenopus* egg extracts as well [4, 5], one could consider the possibility of switching to the frog system. But although *Xenopus* eggs are extremely rich in enzymatic activities and provide an interesting system to examine the mechanism of mismatch correction at a crucial stage of life in vertebrates, the extracts harbor several disadvantages. 1) The overall repair efficiency is significantly lower than that observed in human nuclear extracts. A G/T mismatch, for example, is repaired up to 60% in our *in vitro* system compared to 3-12% repair in *Xenopus* extracts. Furthermore, repair of ^{m}G-containing substrates has never been studied and there is no published information about recognition of this lesion by the *Xenopus* MMR system; 2) Different distributions of the various mismatch recognition efficiencies in *Xenopus* extracts and monkey kidney cells raise the question of relevance to the human system [5, 6]; 3) DNA strand breaks act as signals for the induction and direction of MMR rather than being start or end points for excision and re-synthesis [4], thus differing from the human system [7]. In addition, and probably as a consequence, the repair of mismatches is not strictly biased to the nicked strand as observed in human MMR [4, 7]; 4) The oocyte is provided with a very specialized complement of proteins and cellular components, and it is possible that mismatch repair concentrates predominantly on the correction of DNA replication errors during this stage of development.

5-fluorouracil (5-FU), an antimetabolite widely used in the treatment of colorectal cancer, inhibits thymidylate synthase (TS) and can be incorporated into both RNA and DNA. Although the cytotoxicity is probably due to pleiotropic effects of the drug, DNA incorporation has been suggested to play a major role [8, 9]. We could show that 5-FU/G mismatches are very efficiently processed by both MMR and BER. In the absence of MMR, TDG and UNG, 5-FU/G repair was completely abolished in our *in vitro* system, implying that MBD4 and SMUG1 do not contribute significantly to this repair event. Indeed, SMUG1 turned out to be inactive under standard MMR conditions. Although the enzyme was active in the supplied reaction buffer, it did not show any

activity in the MMR buffer. Since the latter mimics a physiological environment, this finding was very surprising and it would be of interest to find working conditions in order to study all the glycosylases at once. In the absence of both TDG and UNG, MMR processed 5-FU/G mismatches equally well as G/T mispairs. 5-FU/A mismatches, although not recognized in bandshift experiments, were nevertheless processed to a minor extent by the MMR system, as could be shown by visualization of radiolabeled repair DNA synthesis. Interestingly, TDG and UNG reduced the observed MMR processing, indicating a direct competition between the two repair pathways. We do not know how and if TDG and UNG really cooperate *in vivo*. Bandshift experiments with the two glycosylases revealed an interaction between TDG and 5-FU/G- but not 5-FU/A-containing oligonucleotides (data not shown). This is consistent with the idea that 5-FU/A mismatches are recognized and repaired by other glycosylases than TDG, namely UNG or SMUG1 [9, 10]. Our results imply a possible link between the presence or absence of glycosylases, in particular TDG, and the efficiency of 5-FU-based chemotherapies.

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Nationalität	CH
Heimatort	Oberengstringen (ZH)
Geburtsdatum	16. November 1976
Zivilstand	Ledig, keine Kinder Lebenspartner Jürg Unterweger
Konfession	Römisch-Katholisch

Werdegang & Ausbildungen

10.2003-05.2007	Ph.D. am "Institute of Molecular Cancer Research" bei Prof. Josef Jiricny, Universität Zürich-Irchel
10.2006-12.2007	"Höheres Lehramt Mittelschulen": Fachdidaktische Kurse: Fachdidaktik Biologie, Kolloquium für Diplomkandidaten
10.2001-03.2004	Berufsbegleitender Studiengang "Betriebs-ökonom/in KSZH" an der Kaderschule Zürich KSZH (www.kszh.ch)
01.2003-09.2003	Festanstellung im Ressort "Legal & Compliance", Abteilung Ausbildung, Credit Suisse, Zürich
09.2001-12.2002	"Career Start Program" für Hochschul-absolventen, Credit Suisse, Zürich Einsatz 1: Investitionsgüter-Leasing Einsatz 2: Trainerin Lehrlingsausbildung

01.2001-09.2001	Wissenschaftliche Mitarbeiterin bei ESBA-Tech AG, Zürich (www.esbatech.com)
10.1998-08.1999	"Höheres Lehramt Mittelschulen": Lehrveranstaltungen in allgemeiner Didaktik und Jugendpsychologie; Absolvierung der Zusatzprüfung in Anatomie und Physiologie
10.1996-10.2000	Biologie-Studium mit Hauptfach Molekularbiologie und Nebenfach Zoologie an der Universität Zürich-Irchel
04.1996-10.1996	Telefonistin bei der Diener AG, Zürich
08.1991-01.1996	Lehramtsschule (Matura L) an der Kantonsschule Stadelhofen, Zürich

JOBTÄTIGKEITEN IM DETAIL

10.2003-05.2007

Titel meiner Dissertation: *The function of mismatch repair proteins in response to DNA damage caused by chemotherapeutic agents*
Doktorarbeit über die molekulare Wirkung verschiedener Chemotherapeutika im Kampf gegen den vererbbaren Dickdarmkrebs HNPCC (hereditary non-polyposis colorectal cancer). Ein wichtiges Merkmal dieser Krebsform ist das Nichtvorhandensein der Basenfehlpaarungsreparatur (MMR). MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) ist unter anderem für die zytotoxische Methylierung von Guanin (O^6-Methylguanin) verantwortlich, was zur Folge hat, dass die Zelle in Anwesenheit der MMR Apoptose einleitet. MMR-defiziente Tumore sind häufig resistent gegen methylierende Agensien. Das standardmässig in der Klinik eingesetzte 5-Fluorouracil (5-FU) wird sowohl in RNA als auch in DNA inkorporiert und inhibiert zusätzlich die Thymidylat-Synthetase. Der genaue Mechanismus der Zytotoxizität von 5-FU ist aber nicht bekannt. Meine Dissertation untersucht nun biochemisch, wie die oben genannten Basenfehlpaarungen (O^6-mG/T; O^6-mG/C; 5FU/A; 5FU/G) von verschiedenen Reparatursystemen in der Zelle erkannt und repariert werden, mit dem Ziel, den molekularen Mechanismus dieser chemotherapeutischen Mittel zu verstehen und bessere Behandlungsmethoden entwickeln zu können.

01.2003-09.2003

Festanstellung im bankinternen Rechtsdienst "Legal & Compliance", Abteilung Ausbildung (Credit Suisse, Zürich). Nach der Vertretung des Webmasters (Neugestaltung, Pflege und Realisation (Publikation) der internen Web-Seiten) widmete ich mich der Ausbildung neuer Mitarbeiter in diesem Bereich und arbeitete bei der Entwicklung, Durchführung und Auswertung interner Schulungsprogramme mit.

09.2001-12.2002

"Career Start Program" für Hochschulabsolventen (Credit Suisse, Zürich). Von September 2001 bis Juni 2002 hatte ich meinen ersten Einsatz im Bereich "Investitionsgüter-Leasing für Firmenkunden" mit direktem Kundenkontakt. In der verbleibenden Zeit (Juni 2002 - Dezember 2002) bot sich mir die Gelegenheit, als Trainerin in der Lehrlingsausbildung vier Klassen im Bankfach zu unterrichten und ich konnte so während meines Programmes insgesamt 33 Tage Lehrtätigkeit sammeln.

01.2001-09.2001

Wissenschaftliche Mitarbeiterin in der Biotech-Firma ESBA-Tech AG in Zürich. In der Bäckerhefe *S. cerevisiae* werden Antikörper generiert, die später therapeutisch in der Medizin zum Einsatz kommen sollen. Auch das Präsentieren, Auswerten und Analysieren der Resultate, sowie Literatursuche und Mitarbeit beim Schreiben von Publikationen gehörten zu meiner Arbeit. Details unter www.esbatech.com.

10.1996-10.2000

Biologiestudium an der Universität Zürich-Irchel, Hauptfach Molekularbiologie, Grosses Nebenfach Zoologie, Kleines Nebenfach (obligatorisch) Physikalische Chemie. Die Diplomarbeit "The influence of the adenovirus E1A protein on p300/CBP histone acetyltransferase activity and on *in vivo* histone acetylation" wurde unter der Leitung von Dr. Richard Eckner ausgeführt und von Prof. Walter Schaffner, Molekularbiologisches Institut, Universität Zürich-Irchel, begutachtet.

PUBLIKATIONEN

F. Fischer, K. Baerenfaller and J. Jiricny 5-fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems. **(2007)** *Gastroenterology (manuscript submitted)*

P. Cejka, F. Fischer and J. Jiricny Complex interplay of mismatch repair and DNA recombination during the processing of cytotoxic methylation damage. **(2007)** (*manuscript in preparation)*

K. Baerenfaller, F. Fischer and J. Jiricny Characterisation of the "mismatch repairosome" and its role in the processing of modified nucleosides *in vitro*. **(2006)** *Methods Enzymol* **408:**285-303

E. Cannavo, G. Marra, J. Sabatés-Bellver, M. Menigatti, S.M. Lipkin, F. Fischer, P. Cejka and J. Jiricny Expression of the MutL homologue hMLH3 in human cells and its role in DNA mismatch repair. **(2005)** *Cancer Res* **65(23):**10759-66

E. Alvino, D. Castiglia, S. Caporali, R. Pepponi, P. Caporaso, P. M. Lacal, G. Marra, F. Fischer, G. Zambruno, E. Bonmassar, J. Jiricny, and S. D'Atri A single cycle of treatment with temozolomide, alone or combined with O^6-benzylguanine, induces strong chemoresistance in melanoma cell clones *in vitro*: Role of O^6-methylguanine-DNA methyltransferase and the mismatch repair system. **(2006)** *Int J Oncol* **29(4):**785-97

SPRACHEN

Deutsch	Muttersprache
Englisch	Gute Kenntnisse in Wort und Schrift
Französisch	Gute Kenntnisse
Italienisch	Kenntnisse

EDV

Gute Kenntnisse (PC und Mac) in Windows Office 2004 (Word, Excel, Powerpoint), Photoshop und – seit meiner "Webmaster-Tätigkeit" – Grundkenntnisse der Macromedia-Produkte Dreamweaver und Flash

REFERENZEN

Nach Absprache

HOBBIES

M*USIK* – ich spiele seit bald 23 Jahren Klavier und seit 10 Jahren Steeldrums. Zusammen mit zwei Freundinnen (Querflöte und Cello Steeldrums) treten wir an Hochzeiten und verschiedenen Anlässen auf.

M*ORO* – Zu Weihnachten 2005 erfüllte ich mir meinen allergrössten Kindheitstraum und nahm einen "ausgedienten" Greyhound aus einer Auffangstation bei uns auf.